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<b>(21) International Application Number:</b> PCT/CA00/00411 <b>(22) International Filing Date:</b> 14 April 2000 (14.04.00)  <b>(30) Priority Data:</b> 60/129,492 15 April 1999 (15.04.99) US  <b>(71) Applicant (for all designated States except US):</b> CELFOR INC. [CA/CA]; BC Research And Innovation Complex, 3650 Wesbrook Mall, Vancouver, British Columbia V6S 2L2 (CA).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> FAN, Shihe [CA/CA]; 113-3280 E 58th Avenue, Vancouver, British Columbia V5S 3T2 (CA). JANIC, Vesna [CA/CA]; 701-1650 Burnaby Street, Vancouver, British Columbia V6G 1X3 (CA).  <b>(74) Agents:</b> GALE, Edwin, J. et al.; Kirby, Eades, Gale, Baker, Box 3432, Station D, Ottawa, Ontario K1P 6N9 (CA).		<b>(81) Designated States:</b> AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> ENHANCING GERMINATION OF PLANT SOMATIC EMBRYOS BY PRIMING  <b>(57) Abstract</b>  A process of nutrimpriming plant somatic embryos prior to sowing, comprising contacting an imbibed plant somatic embryo with a solution containing one or more dissolved nutrients, preferably including sucrose as one of the nutrients. The invention includes germinated embryos produced by the process, and a method of producing seedlings or full-grown plants incorporating the nutrimpriming step. The method preferably makes use of a growth medium containing the nutrient or nutrients used in the nutrimpriming step.		

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TITLE: Enhancing Germination of Plant Somatic Embryos by Priming

#### TECHNICAL FIELD

This invention relates to the germination of plant somatic embryos. More particularly, the invention relates to a process of treating plant somatic embryos with a nutrimpriming step to enhance subsequent germination of such embryos.

#### BACKGROUND ART

A plant seed is a complete self-contained reproductive unit generally consisting of a zygotic embryo, storage reserves of nutrients in structures referred to as cotyledons, endosperm or megagametophytes, and a protective seed coat encompassing the storage reserves and embryo. In nature, maturation of plant seeds is usually accompanied by gradual loss of water over a period of time to levels between 10-35% moisture content. Once these low moisture levels are achieved, plant seeds can be stored for extended periods.

Germination of zygotic plant seeds is generally triggered by one or more environmental cues such as the presence of water, oxygen, optimal temperature, and light. Seeds germinate by means of a series of events which commence with the uptake of water by a quiescent dry seed and then subsequently proceed through various biophysical, biochemical and physiological events which ultimately result in the elongation of the embryo along its axis.

For the purpose of simplifying discussion of the present invention, the continuous process of seed germination is divided into three phases. Phase one is referred to as imbibition and is characterized by a rapid initial influx of water into the seed. Other significant events occurring in Phase one are the initiation of repair to damage to DNA and mitochondria which may have occurred during seed desiccation and/or the maturation process, and subsequent commencement of protein synthesis facilitated by existing mRNA.

Phase two is characterized by a significant reduction in the rate of water uptake (i.e., imbibition has been completed). This is accompanied by activation or de novo synthesis of enzymes that specialize in hydrolyzing the complex storage reserves of carbohydrates, proteins, and lipids in the embryo and the cotyledons or megagametophytes. The hydrolysis of these complex storage reserves provides the substrates required for the respiration and growth of the zygotic embryos.

Phase three is characterized by a second rapid increase in the rate of water uptake. Water absorbed during Phase three is used primarily for the initiation of meristomatic cell division at the root and shoot apices of the embryo, and for uptake into the cells along the embryonal axis. Water taken up by the axial cells of the embryo applies turgor pressure which results in axial cell elongation. The net effect is that the embryo elongates to the point of protrusion through the seed coat. Protrusion of a shoot or root radicle through the seed coat signifies the completion of germination and the onset of seedling growth and development.

- 10        The speed and success for germination of zygotic seeds can fluctuate considerably depending on various factors such as the residual influence of environmental conditions in which the seed developed and matured, the amount of storage reserve compounds synthesized during the seed maturation process, the duration of storage, and the quality of the storage environment (e.g.,
- 15 temperature and humidity). From a commercial perspective, it is desirable to reduce the risk of germination failure and to ensure that seeds germinate rapidly and uniformly.

      The commercial need for optimum seed germination performance has led to the development of processes known in the art for zygotic seeds as "seed priming". This term may be defined as the "uptake of water to initiate the early events of germination, but not sufficient to permit radicle protrusion, preferably followed by drying" (McDonald, 2000). Four techniques are currently used commercially to accomplish seed priming. These are hydropriming, osmopriming, matriming and pregermination. However, regardless of the method used, the

25 fundamental principles of seed priming are that: (1) the preliminary stages of germination are activated specifically and exclusively through controlling the availability of water to the seeds, and (2) the germination processes initiated through an external priming process are subsequently arrested by a desiccation step.

- 30        One of the problems with commercializing somatic embryo technologies has been a relatively low rate of conversion to seedlings and low seedling vigor when conversion takes place. It would clearly be advantageous to improve such rates of conversion and levels of seedling vigor.

However, a significant additional problem is the current inability to use conventional horticultural ex vitro techniques, practices and environments for the sowing and germinating of plant somatic embryos. The main reason for the difficulties in successfully germinating plant somatic embryos in non-sterile commercial growing environments using conventional propagation practices is that during the initial stages of germination, matured plant somatic embryos cannot produce their own carbon compounds or derive energy from photosynthesis. Furthermore, they lack the presence of their own energy and nutrient sources that are equivalent to storage reserves contained within cotyledons or endosperm or megagametophyte tissues in zygotic seeds. Consequently, an exogenous source of energy in the form of a selected sugar within a culture medium and other nutrients, must be supplied to the plant somatic embryos for successful germination to occur. Such culture media are highly susceptible to invasion by microorganisms which inevitably result in death or interfere with embryo survival and germination. Consequently, sowing and successful germination of plant somatic embryos on culture media must be conducted under strict aseptic conditions.

Although numerous protocols are known for the sowing and germination of somatic embryos and growing them into intact functional seedlings, all of these protocols are dependent on the use of aseptic techniques combined with in vitro systems that must be kept in biological isolation from contaminating microorganisms and fungi until the plant somatic embryos have successfully completed germination and have achieved autotrophy. Consequently, none of these protocols has demonstrated compatibility with conventional horticultural equipment and practices.

Generally, the known protocols for germinating somatic embryos fall into two categories. The first is a category of protocols based on various in vitro methods which generally are comprised of sowing naked, i.e., uncoated, somatic embryos using aseptic techniques, onto sterilized semi-solid or liquid media contained within a solid-support such as a petri dish or a phytatray to facilitate germination under biologically isolated sterile conditions (e.g., US Patent Nos. 5,183, 757; 5,294,549; 5,413,930; 5,464,769; 5,506,136 all of which are herein incorporated by reference) and subsequently, transplanting the germinants into conventional growing systems. The most significant disadvantages of such in

vitro protocols for sowing naked somatic embryos are that (a) each embryo typically must be handled and manipulated by hand for the germination and transplanting steps, and (b) aseptic techniques and culture conditions must be rigorously maintained through to the step of transplanting of somatic germinants  
5 out of the in vitro germination media into horticultural growing media. Although various automation options, including robotics and machine vision, have been assessed for their usefulness in cost-effective reduction or elimination of the extensive hand-handling currently necessary to sow naked embryos (Roberts et al., 1995), no commercial equipment currently exists which can reliably,  
10 aseptically, and cost-effectively perform the in vitro protocols for germination of naked somatic embryos and subsequent transplanting into conventional propagation systems.

The second category of protocols teach encapsulation (generally gel-encapsulation) of somatic embryos (e.g., US Patent Nos. 4,777,762; 4,957,866;  
15 5,183,757; 5,482,857 all of which are herein incorporated by reference) to provide a means by which the embryos can presumably be sown with mechanical devices such as seeders and fluidized drills, into conventional growing systems. However, there are a number of disadvantages with gel-encapsulated somatic embryos. For example, the hydrated semi-solid physical  
20 characteristics of encapsulated embryos make them incompatible for use with conventional seeding equipment currently available for commercial plant propagation, because the semi-solid gel-encapsulated somatic embryos tend to clump together during handling and consequently, are difficult to singulate and dispense. Furthermore, compositions of encapsulated embryos prepared as  
25 taught by the art, clog-up the conventional equipment, and for these reasons, it currently is not possible to sow encapsulated embryos with conventional seeding equipment. Consequently, novel equipment has been developed specifically for delivery of encapsulated somatic embryos into conventional growing systems. Such sowing devices have been reviewed by Sakamoto et al. (1995), but these  
30 devices have only been developed and tested as prototypes. Because of mechanical limitations and the high costs associated with the prototype mechanical seeders developed for sowing encapsulated embryos, none are currently available for commercial acquisition and use.

Another disadvantage with encapsulated somatic embryos is the lack of nutrient availability that is characteristically supplied to zygotic embryos by their attendant endosperm or megagametophyte tissues. Consequently, the encapsulation technology for somatic embryos has been extended to include the incorporation of various nutrients such as sugars, fertilizers, oxygen, into the encapsulation medium (e.g., Carlson & Hartle, 1995; US Patent Nos. 4,583,320; 5,010,685; 5,236,469, all of which are herein incorporated by reference). However, a distinct disadvantage associated with nutrient-amended encapsulated embryos is their susceptibility to microbial invasion during manufacture, storage, and during germination if germinated on non-sterile media.

Furthermore, it must be pointed out that although considerable prior art (e.g., PCT Patent Application WO 94/24847, and US Patent Nos. 5,010,685; 5,236,469; 5,427,593; 5,451,241; 5,486,218 all of which are herein incorporated by reference) teaches methods to manufacture "artificial seeds" consisting of somatic embryos encapsulated in gels, which may or may not be optionally supplemented with nutrients, and which may or may not be encased within a rigid covering, and although the prior art makes references to sowing said artificial seeds ex vitro into germination media comprised of soil or soil-less mixes, the prior art only teaches methods for germinating said artificial seeds in vitro, i.e., on sterilized semi-solid laboratory media. No methods are taught or otherwise disclosed in the prior art for sowing said encapsulated somatic embryos and/or manufactured and/or artificial seed into conventional growing systems using conventional sowing equipment.

However, the most significant disadvantage with all prior art procedures for encapsulating or otherwise coating somatic embryos, is that somatic embryos processed following those protocols typically have, as a consequence, much lower germination vigor and success than corresponding zygotic seeds (Carlson & Hartle, 1995). Carlson and Hartle (1995) concluded that considerable research is still required before "manufactured" or "artificial" seeds based on encapsulation and/or coating of somatic embryos will have practical utility. However, it should be noted that the germination vigor of naked, i.e., un-coated or non-encapsulated somatic embryos produced with methods disclosed in the art and then sown using aseptic technique onto in vitro germination media and subsequently

germinated in sterile conditions, can approximate those of the corresponding zygotic seeds (e.g., greater than 85%) (Gupta & Grob, 1995).

Timmis et. al. (US Patent No. 5,119,588 incorporated herein by reference) teach a method by which plant somatic embryos can be sown into horticultural  
5 containers filled with particulate soil-like substrates. Solutions containing carbon compounds serving as energy sources and other nutrients are added to the substrates before or after the embryos are sown. However, they teach that it is essential that the containers, substrate, nutrient solutions and other components of their system must be biologically sterile. Furthermore, once the somatic  
10 embryos are sown into their system using aseptic techniques, each individual container must be kept biologically separated from the others and from the external environment and must be kept in a sterile condition until the embryo has successfully germinated, formed functional roots and shoots and has accomplished autotrophy. Only after autotrophy has been reached can the  
15 somatic seedlings be removed from the sterile conditions within their system, and then transplanted into a conventional commercial propagation environment.

Even though it is possible to successfully germinate plant somatic embryos on culture media using aseptic techniques and subsequently transplant the germinants into nursery production environments, such methods are labor-  
20 intensive, slow and very costly. Furthermore, somatic embryos obtained in accordance with the prior art are not amenable to the systems and equipment commonly used for commercial production of plant material. Therefore, there is still a need for a practical method of supplying exogenous energy and nutrient sources to plant somatic embryos in a manner that will facilitate and maximize  
25 the ex vitro germination of somatic embryos and the subsequent plant development and growth under non-sterile conditions.



## DISCLOSURE OF THE INVENTION

An object of the invention is to enhance germination of plant somatic embryos.

Another object of the invention is to improve the reliability and  
5 synchronization of plant somatic embryo germination.

Another object of the invention is to facilitate the growth of plants and seedlings from plant somatic embryos.

Another object of the invention is to enable the germination of plant somatic embryos to occur in non-sterile conditions in growing media commonly  
10 used in commercial nursery practice.

According to one aspect of the invention, there is provided a process of priming plant somatic embryos, which comprises contacting mature plant somatic embryos with an aqueous solution containing a dissolved nutrient.

The nutrient is preferably a carbohydrate, e.g. a sugar such as sucrose.  
15 When sucrose is employed as the priming nutrient, it is preferably used at a concentration in an aqueous medium of 6% w/v or less.

According to another aspect of the invention, there is provided a process of nutrimpriming plant somatic embryos prior to germination, comprising contacting imbibed plant somatic embryos with a solution comprising a mixture of two or  
20 more dissolved nutrients.

According to another aspect of the invention, there is provided a method of producing seedlings or full-grown plants from somatic embryos, which comprises nutrimpriming plant somatic embryos by contacting imbibed plant somatic embryos with a solution containing a dissolved nutrient, germinating the  
25 nutrimprimed embryos in a growth medium to form germinants, and maintaining growing conditions to allow the germinants to grow into seedlings or full-grown plants.

According to another aspect of the invention, there is provided a method of producing seedlings or full-grown plants from somatic embryos, which  
30 comprises nutrimpriming plant somatic embryos by contacting imbibed plant somatic embryos with a solution containing a mixture of dissolved nutrients, germinating the nutrimprimed embryos in a growth medium to form germinants, and maintaining growing conditions to allow the germinants to grow into seedlings or full-grown plants.

According to another aspect of the invention, there is provided a method of germinating mature plant somatic embryos, comprising priming imbibed embryos in the presence of a nutrient medium.

According to yet another aspect of the invention, there is provided a  
5 method of producing somatic seedlings, comprising (i) priming imbibed mature plant somatic embryos in a nutrient medium, and (ii) sowing the embryos in a growth medium.

The invention also relates to somatic embryos, seedlings or mature plants produced by the above methods.

10 The somatic embryo is preferably from a tree species, most preferably a gymnosperm, e.g. pine. The process is particularly effective with spruce and pine somatic embryos.

It is an advantage of the present invention, at least in preferred forms, that it can provide a process by which an imbibed somatic embryo can be nutrimprimed  
15 in solutions containing one or more nutrients, then harvested from the nutrimpriming medium, and subsequently sown, germinated and grown *ex vitro* using conventional horticultural and agricultural equipment, containers, growing substrates, and growing environments. Furthermore, the sowing, germination and growing steps can be performed without the use of biologically isolating  
20 enclosures, or sterile media and growing environments. Alternatively, after the nutrimpriming step is completed, primed somatic embryos can be dried and stored for periods of time prior to *ex vitro* sowing and germination

Another advantage of the invention, at least in preferred forms, is that it can provide a process by which the nutrimpriming of somatic embryos followed by  
25 harvesting and subsequent *ex vitro* sowing, germination and growing, can be practiced with a diverse variety of gymnosperm and angiosperm species. Alternatively, harvested nutrimprimed somatic embryos of both gymnosperm and angiosperm species may be desiccated and stored for periods of time prior to *ex vitro* sowing and germination.

30 In preferred forms, the present invention relates to a multi-step process to produce seedlings from mature somatic embryos which begins by imbibing somatic embryos and then placing the imbibed somatic embryos into solutions containing nutrients for periods of time. This first component of the multi-step process is referred to as "nutrimpriming." It has surprisingly been found that

nutriprimed somatic embryos can be sown *ex vitro* using various non-sterile methods into a wide variety of horticultural nursery containers filled with various types of non-sterile growing mixes commonly used in commercial horticultural and agricultural plant propagation. Once placed into conventional non-sterile  
5 nursery propagation systems and using conventional horticultural growing practices, the nutriprimed somatic embryos will germinate and grow into fully functional plants. Furthermore, we have surprisingly found that nutripriming solutions can be applied using conventional non-sterile horticultural equipment to facilitate and enhance the germination of nutriprimed somatic embryos.

10 We have also surprisingly discovered that nutriprimed somatic embryos can be desiccated to moisture contents in the range of 10-76%. Furthermore, we have discovered that desiccated nutriprimed somatic embryos can be stored for extended periods of time without significant declines in physiological integrity or germination potential. We have also discovered that desiccated nutriprimed  
15 somatic embryos are amenable for sowing with conventional seeding equipment into conventional plant propagation media for germination and further growth and development using conventional non-sterile plant propagation practices.

Consequently, the multi-step process of at least preferred forms of the present invention includes, but is not limited to, the steps of imbibing somatic  
20 embryos, nutripriming said imbibed somatic embryos, placing said nutriprimed somatic embryos into a state of physiological dormancy, sowing said nutriprimed physiologically dormant somatic embryos onto or into conventional horticultural germination substrates, propagating said sown nutriprimed somatic embryos in environmental conditions manipulated to facilitated imbibition, germination, and  
25 development into complete seedlings possessing shoots and roots.

There are several advantages inherent with the use of the process of the invention. For example, one advantage of nutripriming plant somatic embryos is that they show exceptional vigor during germination and subsequent development into complete seedlings possessing shoots and roots. Furthermore,  
30 desiccated nutriprimed somatic embryos are particularly useful for preserving the physiological viability of the embryos during extended storage prior to sowing and germination. Yet another advantage of nutripriming somatic embryos is that they can be sorted according to size, length and shape to facilitate production of more uniform crops after sowing, germination and growth.

A key advantage of the present process is that once the nutripriming step is completed, all subsequent components of the multi-step process can be practiced in conventional plant propagation environments without the need for aseptic handling processes or for sterile growing environments. More specifically, aseptic procedures, and sterile or sanitized equipment and germination/growing environments are not required for successful *ex vitro* sowing and germination of nutriprimed somatic embryos and their subsequent development into complete functional seedlings, thus enabling the entire sowing, germination, and growing steps to be performed, if so desired, in commercial plant propagation or greenhouse or nursery growing facilities.

Another advantage is that the nutriprimed somatic embryos can be sown with conventional seeding equipment such as but not restricted to, vacuum-drum seeders, fluid-drill seeders or needle-jet seeders.

A further advantage is that commonly used horticultural and agricultural products such as, but not restricted to, soil-less seedling mixes or rock wool or foams, can be used as the supports onto which the nutriprimed somatic embryos are sown and subsequently germinate into and penetrate with their roots.

Most preferably, a nutrient (ideally the same nutrient as the one used in the nutripriming step) is also incorporated into said growth medium.

Yet a further advantage is that if necessitated by the conditions in the commercial growing environments, existing commercial pesticide products such as, but not restricted to fungicides, bactericides, antibiotics, nematicides, insecticides and the like, which are registered for use with the plant species from which the somatic embryos are produced, can be applied to the sown nutriprimed somatic embryos per label instructions for effective pest control, or alternatively, applied to the growing substrates prior to sowing the somatic embryos.

Another advantage is that exogenous nutrients necessary for successful somatic embryo germination and growth can be applied via the various numerous methods commonly used in commercial horticulture, said methods including but not restricted to misting, fogging, spraying, watering and drenching.

Furthermore, said exogenous nutrients can be applied in conjunction with conventional horticultural fertigation practices.

This invention includes the above objects and features taken alone and in combination. These and other features, objects and advantages of the present invention will become more apparent with reference to the following description.

## 5 DEFINITIONS

A number of terms are known to have differing meanings when used in literature describing this art. The following definitions are believed to be ones most generally used in the fields of botany, plant somatic embryogenesis, and are consistent with the usage of the terms in the present specification.

10 These definitions will assist in the understanding of this detailed description.

"ABA" is abscisic acid, a plant growth regulator.

"Autotrophic" refers to the stage of plant development when the photosynthetic organelles and related enzymes and biochemical pathways are fully functional and capable of converting light energy, atmospheric carbon dioxide and water  
15 into the pre-requisite carbohydrates (e.g., glucose) necessary to sustain further plant growth and development.

"BA" is benzyl adenine, a cytokinin-type of plant growth regulator. The main physiological effect of BA is to stimulate meristomatic cell division.

"Clone" when used in the context of plant propagation refers to a collection of  
20 individuals having the same genetic constitution, and are produced from a culture that arises from an individual explant.

"Embryogenic culture" is a plant cell or tissue culture capable of forming somatic embryos and regenerating plants via somatic embryogenesis.

"Endosperm" is haploid nutritive tissue of angiosperm seed, of maternal origin,  
25 within which the angiosperm zygotic embryos develop.

"Explant" is the organ, tissue or cells derived from a plant and cultured *in vitro* for the purposes of starting a plant cell or tissue culture.

"GA" is gibberellin, a group of related growth regulator isomers (e.g., GA<sub>3</sub>, GA<sub>4</sub>, GA<sub>7</sub>) that is naturally synthesized as a normal part of plant metabolism. The main physiological effect of GA is to stimulate elongation of individual plant cells.

Exogenous applications of GA can be used to stimulate, manipulate and

5 accelerate the initiation and growth of shoots and roots

"Germination" is the three-phase series of events which commences with the uptake of water by a quiescent dry seed and proceeds through various biophysical, biochemical and physiological events which result in the elongation of the embryo along its axis and ultimately concludes with the protuberance of a

10 root or shoot radicle through the seed coat. Seed germination occurs in three phases. Phase one is characterized by a rapid initial influx of water into the seed accompanied by the initiation of repair to damaged DNA and mitochondria. Phase two is characterized by a significant reduction in the rate of water uptake accompanied by activation or *de novo* synthesis of enzymes that specialize in  
15 hydrolyzing the complex storage reserves of carbohydrates, proteins, and lipids in the embryo and the cotyledons or megagametophytes. The hydrolysis of these complex storage reserves during Phase two provides the substrates required for the respiration and growth of the zygotic embryos. Phase three is  
20 characterized by a second rapid increase in the rate of water uptake which is used primarily for the initiation of meristomatic cell division at the root and shoot apices of the embryo, and for uptake into the cells along the embryonal axis. The process of germination is complete when the embryo has elongated to the point of protrusion through the seed coat.

"Hydropriming" is a seed priming process by which seeds are misted or soaked in  
25 water and then dried back before they complete the germination process.

"IAA" is indole-acetic-acid, a auxin-type growth regulator naturally synthesized as normal part of plant metabolism. The main physiological effect of IAA is to stimulate meristomatic cell division. Exogenous applications of IAA can be used to stimulate, manipulate and accelerate the initiation and growth of shoots and

30 roots.

"IBA" is indole-butyric-acid, a chemical analog of IAA. IBA can be used to affect the initiation of roots and shoots in the same manner as exogenous applications of IAA.

"Imbibition" is the absorption and/or adsorption of water by certain colloids present in seeds or embryos, which results in the swelling of the tissues and activation of enzymatic and physiological processes.

"Line" is another term for "clone".

"Matrimpriming" refers to the use of solid carriers with low matric water potentials to control the rate and/or the amount of water absorbed during a seed priming process.

"Megagametophyte" is haploid nutritive tissue of gymnosperm seed, of maternal origin, within which the gymnosperm zygotic embryos develop.

"Microdroplet" is small molecule of water or water-based solution contained within the fine spray produced by applying pressure to a drop of water or a water-based solution.

"Nutrients" are the inorganic micro- and macro-minerals, vitamins, hormones, organic supplements, and carbohydrates necessary for culture growth and somatic embryo germination.

"Nutrient solution" is water containing a dissolved nutrient or mixture of nutrients.

"Nutrimpriming" refers to a process of exposing a plant somatic embryo to a nutrient solution during the Phase two period of germination for a period of time sufficient to enable the embryo to absorb mineral and organic nutrients necessary to complete the Phase two and Phase three steps of germination.

"Osmopriming" refers to a method of soaking seeds in aerated osmotica of low water potential to control the rate and/or the amount of water absorbed during a seed priming process.

"Physiological dormancy" refers to the cessation of the normal metabolic processes, i.e., anabolism and catabolism, that are inherent in plant growth and development, in a manner that does not negatively affect viability.

"Pregermination" is a seed priming process which allows water availability to  
5 seeds to the point of shoot or root radicle protrusion from the seed coat, before the desiccation step is applied.

"Seed priming" refers to a process which controls and manipulates the water availability to seeds to initiate and affect the early events of germination, but not sufficient to permit radicle protrusion, which is subsequently followed by drying.

10 "Somatic embryogenesis" is the process of initiation and development of embryos *in vitro* from somatic cells and tissues.

"Somatic embryo" is an embryo formed in vitro from vegetative (somatic) cells by mitotic division of cells. Early stage somatic embryos are morphologically similar to immature zygotic embryos; a region of embryonal cells subtended by  
15 elongated suspensor cells. The embryonal cells develop into the mature somatic embryo.

"Zygotic embryo" is an embryo derived from the sexual fusion of gametic cells.

#### BEST MODES FOR CARRYING OUT THE INVENTION

20 In a preferred form, the present invention is generally a multi-step germination process for plant somatic embryos which enables the use of conventional horticultural practices, equipment and facilities, said process comprising but not restricted to, some or all of the following sequential steps:

1. Initiating Phase one of the germination process by imbibing desiccated  
25 mature plant somatic embryos. It is preferable that the rate of imbibition during Phase one is controlled through the use of a process such as matrimpriming. It is also preferable that this step is performed using aseptic techniques and sterile conditions.



2. Once the Phase one germination step, i.e., imbibition, has been completed, the imbibed plant somatic embryos are transferred to a vessel containing a liquid nutrimpriming solution. The nutrimpriming solution must contain at least one source of carbohydrate. Although the preferred carbohydrate is sucrose, preferably in the range of 3-6% (w/v), this invention can be practiced with sugars such as fructose, glucose, maltose, galactose, mannose, lactose and the like. Furthermore, the nutrimpriming solution may contain, if so desired, a mixture of two or more carbohydrates. If carbohydrates other than sucrose, or if mixtures of carbohydrates, are used in the nutrimpriming solution, then the appropriate concentrations of each carbohydrate should be determined in advance by the use of rate-selection studies. The design and performance of such rate-selection studies are known to those skilled in this art. Another key feature of the present invention is that in addition to carbohydrates, the nutrimpriming solutions may also contain, if so desired, other types of nutrients which may further facilitate the various biochemical and physiological processes occurring during germination Phases two and three. Such nutrients include but are not restricted to inorganic minerals, vitamins and hormones. A non-limiting example of how this can be practiced is by adding to a solution containing sucrose in the range of 3-6% (w/v), a mixture of mineral nutrients formulated to deliver but not restricted to 454 mg/l nitrogen, 81 mg/l phosphorus, 704 mg/l potassium, 50 mg/l calcium, 39 mg/l magnesium, 193 mg/l sulfur, 3 mg/l manganese, 0.5 mg/l zinc, 89 mg/l chlorine, 3 mg/l iron, 0.7 mg/l iodine, 0.6 mg/l boron, 0.01 mg/l molybdenum, 0.01 mg/l cobalt, and 0.01 mg/l copper. Furthermore if so desired, IBA a plant growth regulator, may be added alone at a concentration of 0.1  $\mu$ M/l or in combination with one or both of GA and BA, each at a concentration of 0.1  $\mu$ M/l. Also, Ascorbic acid (a.k.a. vitamin C) may be added if so desired, at a concentration in the range of 10–1000  $\mu$ M/l. Furthermore, if so desired, pest control products such as antibiotics or fungicides may be added to the nutrimpriming solution. A non-limiting example is the addition of benlate (0.1 g/l) and/or ampicillin (0.1 g/l). It is preferable during this step that the nutrimpriming solutions are sterilized prior to addition of the somatic embryos, and that

aseptic technique is used when adding embryos to the nutriming solution.

3. The imbibed embryos are nutrimed in the nutriming solution for a period of time preferably ranging from 6 hours to 168 hours, more preferably in the range of 48 to 96 hours and most preferably between 72 to 96 hours. It is also preferable throughout the nutriming process, that the embryos are suspended within the nutriming solutions and are kept in constant motion. A non-limiting example of how this might be accomplished is by securing the vessels containing the embryos and nutriming solutions onto a shaker table which is revolving at a rate in the range of 10-120 rpm, preferably in the range of 30-60 rpm. It is possible to practice the nutriming step in either the presence or absence of light. Since it is known in the art that zygotic seeds of certain plant species will germinate only in the dark while zygotic seeds of other plant species require light for successful germination, those skilled in the art will be able to determine if the nutriming step should be illuminated for the plant somatic embryos with which they wish to practice this invention. It is preferable that the contents of the nutriming vessels are maintained in a sterile condition during the nutriming process.
4. Sowing the nutrimed plant somatic embryos into nursery containers containing a three-phase conventional horticultural growing substrate, said three phases comprising solids, liquids and air. Commencing with this step, aseptic technique and sterile conditions are not required to successfully practice this invention.
5. Placing the nursery containers sown with nutrimed plant somatic embryos, into a conventional plant propagation environment in which light, temperature, atmospheric humidity, and moisture content of the rooting substrate can be controlled and manipulated to enable and facilitate the re-germination of the somatic embryos and their further development into seedlings.

6. If so desired, supplying an aerosol in the form of a mist or spray, to the surface of the nursery containers sown with somatic embryos, said aerosol containing the necessary carbohydrate compounds required to sustain and facilitate completion of the Phase three germination processes of the somatic embryos.
  7. Supplying in the forms of an aerosol and/or a liquid suspension and/or a liquid solution, the micro- and macro-mineral elements required to sustain and facilitate completion of the Phase three germination processes of the somatic embryos and their subsequent development into seedlings.
  8. Adjusting as required during completion of the somatic embryo germination processes and subsequent development into seedlings, the ambient light intensity and diurnal photoperiod, temperature, atmospheric humidity and other such factors may be adjusted as required during somatic embryo germination and their conversion into fully functional seedlings.
- Alternatively, at the completion of step 3, nutrimed embryos may be removed from the nutriming solutions and desiccated to a moisture content preferably in the range of 10% to 75%, more preferably in the range of 15% to 50% and most preferably in the range of 20% to 25%. For example, the embryos may be desiccated by pouring the nutriming solutions with the nutrimed embryos onto filter paper in a vacuum filter apparatus for the removal of the excess solution; other methods of desiccation are known to those skilled in the art. The filter paper with primed embryos on their surfaces can then be placed into "flow-through" desiccation chamber for a period of 3-96 hr, preferably 12-24 hr. After desiccation, the dried nutrimed embryos can be stored in sealed, plastic-lined pouches. Although desiccated nutrimed embryos can be stored at ambient temperatures, it is preferable that they are stored at refrigerated temperatures, e.g., 2 to 10°C, and most preferably, frozen e.g., -20 to -80°C. Although it is preferable to use aseptic technique during the desiccation and storage of nutrimed embryos, it is not essential for the successful practice of this invention. Desiccated nutrimed embryos can be removed from storage and sown *ex vitro* commencing with step 4 above.

A key feature of this novel process, at least in its preferred forms, is that special hygienic and/or aseptic and/or sterile handling methods and/or equipment and/or facilities are not required to successfully handle, sow and germinate wet or desiccated nutriprimed plant somatic embryos. Accordingly, steps 4 through 8  
5 may be carried out in non-sterile, unhygienic and/or septic conditions, i.e., those types of conditions typically encountered in conventional horticultural plant propagation environments.

Although the nutriprimed somatic embryos can be sown with all types of conventional seeding equipment used for sowing zygotic seeds, it is preferred to  
10 use equipment that dispenses singulated seed into multi-chambered nursery containers, commonly referred to as miniplug trays or cell-packs, said containers commonly used to produce plant plugs which can be mechanically transplanted into larger containers or into field-growing environments.

A key feature of the present invention, at least in its preferred forms, is  
15 that the sowing and propagation of nutriprimed somatic embryos can be practiced with a wide variety of non-sterilized growing substrates commonly used in conventional plant propagation. The preferred growing substrate is peat-based and has been formulated specifically for germination of zygotic seed and is exemplified by mixtures such as (a) 100% short-fiber peat product polymerized  
20 with a water-binding polymer, supplied by companies such as Grow Tech Inc. (San Juan Bautista, CA USA), Preforma Inc. (Oberlin, OH USA), (b) 15.2 cu.ft of peat, 8 cu.ft. of vermiculite, 680 grams of dolomite lime, and 300 grams of Micromax®, and (c) 16.2 cu.ft. of peat, 6.75 cu.ft. perlite, 4 cu.ft. vermiculite, 6 kilograms of dolomite lime, 1.5 kilograms of gypsum, 375 grams of potassium  
25 phosphate, 250 grams Micromax®, and 35 grams of wetting agent. Alternatively, commercially formulated mixes such as PRO-MIX-G® or PRO-MIX-PGX® (Premier Peat Moss Ltd. Montreal, PQ, Canada), Sunshine Mix # 3 (Sun-Gro Horticulture Inc., Hubbard, OR, USA), and Redi-Earth® (The Scotts Co., Marysville, OH, USA) can also be used with the present invention. It is preferred  
30 that the peat-based growing substrate is moistened to a moisture content in the range of 59-75% and then dispensed into multi-chambered trays commonly used for production of plant plugs. Although examples of such trays include styrofoam #252 miniplug trays manufactured by Beaver Plastics Inc (Edmonton, AB,

Canada) and hard plastic #288 or #512 miniplug trays manufactured by TLC Polyform Inc (Plymouth MN, USA, 55441), the present invention can be practiced with other such multi-chambered trays, or alternatively, with individual pots. It should be noted that the practice of the present invention is not restricted to peat-based mixtures, but also includes other substrate such as Jiffy-7 peat plugs, composted or shredded coconut husk fibres commonly referred to as "cor" or "coir" (1993 Crystal Co., St. Louis, MO, USA), extruded foams such as Oasis® (Smithers-Oasis Ltd., Kent, OH, USA), rock wool (Rockwool International A/S, Hovedgaden 584, DK-2640, Denmark) and the like. Regardless of the rooting substrate chosen, its physical characteristics should enable development and maintenance of a high relative humidity in the gaseous phase, i.e., in excess of 75% RH, within the substrate while minimizing saturation of the substrate with the liquid phase.

After the pre-germinated somatic embryos are sown onto the surfaces of the rooting substrates, if so desired, the embryos may be covered with a thin layer of additional rooting substrate that may be comprised of the same material underneath the embryos or alternatively, with a different type of material. One non-limiting example is sowing the pre-germinated embryos onto PRO-MIX-PGX® medium, then overlaying the embryos with a thin layer of coconut husk fibres.

Nursery containers sown with pre-germinated somatic embryos are preferentially placed into a conventional plant propagation environment wherein the conditions are within but not limited to the ranges of temperatures of 15-35°C, relative humidities of 75-100%, light intensities of 10-500 foot candles, and diurnal cycles of 6h day/18h night - 22h day/2h night.

It is preferable to maintain a very high level of atmospheric humidity around the nursery containers sown with pre-germinated somatic embryos, i.e., greater than 90% RH, for the first 3-7 days after sowing to facilitate somatic embryo imbibition and germination. A number of methods can be used to maintain the atmospheric humidity at these levels including but not restricted to placing the containers in a greenhouse environment with misting or fogging equipment which is deployed at controlled intervals, placing the containers in a fogging or misting tent or chamber, placing clear plastic domes over the nursery containers and then removing domes periodically to mist or fog the sown embryos and replacing the domes immediately thereafter. Another non-limiting

method is to provide a space ranging between 2 mm and 10 mm above the surface of the rooting substrate onto which the embryos are sown and the top of the container, and then covering the top of the nursery container with a plastic film which is removed to enable misting or fogging of the sown embryos and then immediately replaced. After somatic embryo germination is established as evidenced by development of epicotyl and root structures, the germinants can be weaned from the high relative humidity environments and integrated into conventional nursery cultural practices by gradually reducing the amount of misting/fogging applied and/or by extending the periods of time between the misting or fogging steps.

It is preferable to maintain the sown pre-germinated embryos in a high relative humidity environment, i.e., greater than 90% RH, for a period of, but not restricted to, 3 - 7 days after sowing to facilitate embryo imbibition, prior to supplying exogenous nutrients required for embryo germination.

Another key feature of the invention, at least in its preferred forms, is that the exogenous nutrients, including but not restricted to carbohydrates and minerals, required for successful somatic embryo re-germination and subsequent growth and development can be applied as aerosols. The nutrient solutions can be applied with, but not restricted to, conventional misting and/or fogging equipment. Although, the nutrients can be applied individually or combined into one solution, it is preferred to supply the carbohydrates as one solution and the remaining nutrients as a separate solution. A non-limiting example of how this can be practiced is by applying a 3% w/v sucrose solution as a mist to the surface of the growing substrate containing a sown pre-germinated embryo, and then applying at a later time, a solution containing a mixture of mineral nutrients formulated to deliver 454 mg/l nitrogen, 81 mg/l phosphorus, 704 mg/l potassium, 50 mg/l calcium, 39 mg/l magnesium, 193 mg/l sulfur, 3 mg/l manganese, 0.5 mg/l zinc, 89 mg/l chlorine, 3 mg/l iron, 0.7 mg/l iodine, 0.6 mg/l boron, 0.01 mg/l molybdenum, 0.01 mg/l cobalt, and 0.01 mg/l copper. Alternatively, the macronutrients can be supplied as a commercial formulation such as but not restricted to PlantProd® Plant Starter Fertilizer 10-52-10 (nitrogen-phosphate-potassium) or PlantProd® Forestry Seedling Starter 11-41-8 (nitrogen-phosphate-potassium) (Plant Products Ltd., Brampton, ON, Canada).

An alternative non-limited means of supplying exogenous nutrients to pre-germinated somatic embryos sown onto three-phase growing media within nursery containers is to irrigate or "drench" the media with nutrient solutions formulated as previously described.

5 Since microorganisms such as fungi, bacteria, yeast, and algae, are ubiquitous in conventional plant propagation substrates, equipment, containers and growing environments, a wide variety of chemical and biological pesticide products are available to control and eradicate plant pathogens. It has surprisingly been found that aseptic handling procedures and sterilized growing  
10 substrates, nursery containers and environments are not required to successfully germinate and grow plant somatic embryos. Indeed, the invention can be practiced in conventional plant propagation environments using only the standard commercial methods of hygiene. Furthermore, we have surprisingly found that pesticides such as Benlate®, Rovril®, Trumpet® and the like, which registered for  
15 pest control in plant crops, can be used in conjunction with somatic embryos pre-germinated and subsequently sown with the present novel multi-step procedure.

The following Examples are provided to further illustrate the present invention and are not to be construed as limiting the invention in any manner.

20

#### EXAMPLE 1

##### **Germination of nutriprimed interior spruce somatic embryos on a support with and without sucrose supplements**

25 The objective of this study was to determine the effects of nutripriming treatments on the subsequent germination of interior spruce somatic embryos.

Post-HRHT (HRHT is a high-relative humidity treatment described by Roberts et al., 1993) somatic embryos of interior spruce genotype 1-1278 and 107-1917 were divided into groups of 80 embryos. Each group was separately  
30 placed into a 250-ml baffled flask containing 25 ml of m24GMD media (refer to Table 1.1 for the nutrient composition of m24GMD medium) with one of the following sucrose levels: 1, 2, 3, 4% (w/v). The flasks, after being stoppered with a stopper made from cotton and cheesecloth, were placed on a shaker and continuously shaken at 60 rpm and illuminated at  $30\text{--}40\text{ mol m}^{-2}\text{s}^{-1}$  at the liquid  
35 surface height. The light was provided by two 25 W fluorescent bulbs. Ambient

temperatures generally varied between 20-23°C according to measurements.

The embryos were nutrimprimed for 3 days under these conditions.

After nutrimpriming was completed, the 80 embryos in each treatment were divided into 4 groups of 20 embryos. Each group was sown into a germination  
5 box as a replicate of respective treatments. The germination boxes were Sigma™ polycarbonate boxes. Each box contained 150 ml of horticulture coarse vermiculite and 120 ml m24GMD media without sucrose. Before use, the boxes were autoclaved for 20 minutes in a liquid cycle.

To prevent systematic errors that might have occurred had the  
10 experimental units not been randomized, each of the germination boxes housed a replicate from 4 different priming treatments. The assignment of the replicates into the germination boxes was facilitated using a random number table.

Nutrimprimed embryos were hand-sown into the germination boxes. After sowing, the germination boxes were sealed twice with Parafilm™. Embryos were  
15 germinated and grown for one week under 50-70  $\mu\text{mol m}^{-2}\text{s}^{-1}$  photosynthetic photon flux of 16-hour photoperiod at an air temperature of 16°C. Then, the germinants were harvested and photocopies of the individual germinants were made. From these photocopies, germinant length was measured to the nearest 1 mm with a graph paper reproduced on a transparency.

20 The results are summarized in Table 1.2, from which the following observations were made:

- 1) After one week of germination on a vermiculite support without a sucrose supply, no visible changes were observed for the "control" embryos of interior spruce lines 1-1278 and 107-1917
- 25 2) All nutrimprimed primed somatic embryos, regardless of nutrimpriming treatment, became green in colour with elongated epicotyls. Embryos nutrimprimed in 3 and 4% w/v sucrose m24GMD elongated more than those primed in 1 and 2 % w/v sucrose m24GMD with an exception in genotype 107-1917 whose germinants were the longest after priming in  
30 2% w/v sucrose m24GMD.
- 3) None of the embryos sown in the germination boxes produced roots.

In summary, nutrimpriming priming treatment before germination is beneficial, but after sowing, root production requires an additional external sucrose supply.



**Table 1.1: Nutrient composition of m24GMD medium. All weights are expressed in mg l<sup>-1</sup>.**

Chemical	m.w.	wt.	N	P	K	Ca	Mg	S	Mn	Zn	Cl	Cu	Fe	B	Mo	Co	I	Na
K <sub>2</sub> SO <sub>4</sub>	174.2	275.0			123.4			50.5										
CaCl <sub>2</sub> ·2H <sub>2</sub> O	147.0	183.77				50.1					88.6							
KNO <sub>3</sub>	101.1	2106.5	291.7		814.6						253.2							
NH <sub>4</sub> Cl	53.49	382.1	100.0															
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	132.1	677.0	143.5					164.0										
MgSO <sub>4</sub> ·7H <sub>2</sub> O	246.5	394.38					38.9	51.3										
NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub>	115.0	1353.8	164.8	364.6														5.24
NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O	156.0	35.5		7.1				1.6	2.75									
MnSO <sub>4</sub> ·4H <sub>2</sub> O	223.1	11.18						0.3		0.46								
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	287.6	2.04										0.006						
CuSO <sub>4</sub> ·5H <sub>2</sub> O	249.7	0.025																
KI	166.0	0.88			0.2											0.007	0.67	
CoCl <sub>2</sub> ·6H <sub>2</sub> O	202.5	0.024									0.004							
H <sub>3</sub> BO <sub>3</sub>	59.8	3.09												0.56				
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	241.9	0.024													0.01			0.002
FeSO <sub>4</sub> ·7H <sub>2</sub> O	278.0	13.90											2.79					
Na <sub>2</sub> EDTA	372.2	18.61																1.15
Total amount			700	371.6	938.2	50.1	38.9	267.6	2.75	0.46	341.8	0.006	2.79	0.56	0.01	0.007	0.67	6.39
Rel. proportion			100	53	134	7	6	38										

**Notes:** 1) NO<sub>3</sub><sup>-</sup> - N: 291.7 mg l<sup>-1</sup>,2) NH<sub>4</sub><sup>+</sup> - N: 408.3 mg l<sup>-1</sup>,3) The NH<sub>4</sub><sup>+</sup> - N/NO<sub>3</sub><sup>-</sup> - N ratio is 1.4

4) [N] = 50 mM; [P] = 12 mM; [K] = 24 mM.

**Table 1.2:** Germinant length after one week growth on vermiculite support without sucrose supply

Genotype	Nutripriming Treatment	Shoot Length (mm)
1-1278	HRHT	3.2±0.1
	1% w/v sucrose	5.8±0.3
	2% w/v sucrose	5.8±0.3
	3% w/v sucrose	7.0±0.3
	4% w/v sucrose	7.3±0.3
107-1917	HRHT	4.3±0.2
	1% w/v sucrose	7.8±0.3
	2% w/v sucrose	11.5±1.7
	3% w/v sucrose	8.4±0.3
	4% w/v sucrose	8.2±0.5

5

**EXAMPLE 2****Effects of sucrose levels in nutripriming solutions and nutripriming duration on the germination and growth of interior spruce somatic embryos**

10

The objective of this study was to determine the optimal sucrose level and nutripriming duration combination(s) that provide the best germination and growth of interior spruce somatic embryos.

Post-HRHT somatic embryos of interior spruce genotype 143-2695 were divided into groups of 60 embryos. Each group was separately placed in a 250-ml baffled flask containing 25 ml of m24GMD media with one of the following sucrose levels: 1, 2, 3, or 4% (w/v). Embryos were nutriprimed for 5 minutes (for the 2, 3, and 4% w/v sucrose levels only), 3 hours, 1, 2, 3, 4 or 5 days under conditions as specified in Example 1. This created various sucrose level x nutripriming duration combinations.

At the end of the nutripriming treatments, 10 embryos were randomly selected from each nutripriming treatment and measured for length. Then, the 60 embryos in each nutripriming treatment were divided into 4 groups of 15 embryos. Each group was sown into a germination box as a replicate of respective treatments. Each germination box contained 150 ml vermiculite and 120 ml 3% w/v sucrose m24GMD media and housed a randomly assigned replicate from 4 different nutripriming

25

treatments. After sowing, embryos were germinated and grown for two weeks in a germination room under conditions as described in the previous example, after which, the germinants were harvested and measured.

The results are summarized in Tables 2.1-2.4, from which the following  
5 conclusions were made.

- 1) After two days in nutrimpriming treatment, somatic embryos started to elongate. The elongation was up to nearly 65% greater than the control treatments not receiving nutrimpriming. The elongation was similar in all four levels of sucrose nutrimpriming treatment (Table 2.1).
- 10 2) Hyperhydric rates increased with nutrimpriming duration and sucrose levels (Table 2.2).
- 3) Shoot growth of the germinants was stimulated by nutrimpriming treatments. The best growth occurred across all 4 sucrose levels when embryos were nutrimprimed for 2 days (Table 2.3).
- 15 4) Although root growth of the germinants seemed to be optimal when embryos were nutrimprimed for five days, the differences were small among all the nutrimpriming duration x sucrose levels (Table 2.4).
- 6) These results indicate significant interactions between nutrimpriming duration and sucrose levels in the nutrimpriming solution. Optimal germination and growth  
20 could be achieved by (a) combining lower sucrose levels in the nutrimpriming solutions with a longer period of nutrimpriming duration, or (b) higher sucrose levels in the nutrimpriming solutions would enable a shorter nutrimpriming duration.

**Table 2.1** Effects of nutrimpriming treatments on germinant length (mm)

Nutrimpriming period (days)	Control	1% w/v sucrose	2% w/v sucrose	3% w/v sucrose	4% w/v sucrose
	3.10±0.09				
1		3.23±0.15	3.13±0.16	3.06±0.19	3.0±0.15
2		3.44±0.16	3.41±0.12	3.47±0.21	3.44±0.15
3		3.99±0.20	4.16±0.21	3.9±0.20	4.02±0.10
4		5.21±0.22	4.46±0.20	4.18±0.16	4.17±0.19
5		4.8±0.19	4.35±0.30	4.39±0.33	4.63±0.19

5

**Table 2.2** % hyperhydricity rates after a two-week germination period.

Nutrimpriming Period	1% w/v sucrose	2% w/v sucrose	3% w/v sucrose	4% w/v sucrose
Control	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
3 hr	1.7±1.7	5.0±5.0	9.6±3.2	0.0±0.0
1 day	8.3±3.2	3.3±3.3	6.3±2.6	17.7±6.4
2 day	8.3±8.3	4.6±2.8	17.5±8.5	8.3±5.0
3 day	11.7±5.7	11.7±3.7	16.0±9.5	27.9±6.7
4 day	16.7±7.9	56.7±3.3	59.3±6.6	69.1±6.0
5 day	29.2±6.9	48.2±7.4	71.7±9.2	76.7±6.4

**Table 2.3** Shoot length after a two-week germination period (mm).

Nutripriming duration	1% w/v sucrose	2% w/v sucrose	3% w/v sucrose	4% w/v sucrose
Control	11.4±0.4	14.5±0.5	14.2±0.4	16.0±0.5
3 hr	18.7±0.5	16.8±0.5	16.8±0.6	16.7±0.5
1 day	17.2±0.5	15.9±0.3	17.3±0.5	16.6±0.5
2 day	18.4±0.6	17.5±0.5	18.4±0.6	18.0±0.6
3 day	16.5±0.5	17.0±0.4	16.6±0.4	16.1±0.5
4 day	17.4±0.6	16.2±0.5	15.0±0.3	16.0±0.3
5 day	20.9±0.6	16.3±0.5	14.4±0.4	15.6±0.4

5

**Table 2.4** Root length measured at the end of two weeks of germination (mm)

Nutripriming period	1% w/v sucrose	2% w/v sucrose	3% w/v sucrose	4% w/v sucrose
Control	6.1±0.4	6.9±0.4	5.3±0.5	6.7±0.4
3 Hr	8.0±0.4	8.2±0.5	5.8±0.4	6.3±0.4
1 day	7.2±0.4	6.0±0.4	6.0±0.5	7.2±0.4
2 day	6.5±0.4	6.8±0.3	7.7±0.5	6.1±0.4
3 day	7.1±0.4	6.8±0.3	6.8±0.4	5.4±0.4
4 day	7.4±0.5	7.1±0.5	5.7±0.4	7.3±0.4
5 day	8.5±0.3	8.9±0.5	6.8±0.5	8.0±0.5

10

**EXAMPLE 3****Effects of nutriming solution pH on the germination and growth  
of interior spruce embryos**

5

The objective of this study was to determine the optimal pH or pH range of the nutriming solutions that does not negatively affect the germination of nutrimed somatic embryos

- 10 Post-HRHT somatic embryos of interior spruce genotype 4-2809 were divided into groups of 60 embryos and nutrimed in 2% w/v sucrose m24GMD media for 3 days. The nutriming solutions had pHs of 5.0, 5.5, 5.8, 6.0, 6.2, 6.5, 6.8, and 7.0 before autoclaving. The pH of each solution was re-measured 24 hours after autoclaving, just prior to and after the nutriming treatments.
- 15 After nutriming, the embryos were sown into germination boxes and germinated in a germination room for two weeks as described in previous experiments. The germinants were then harvested and measured.

- Autoclaving caused slight decreases in the pH of all nutriming solutions (Table 3). After the 3-day nutriming period, there were further declines in the
- 20 pH of all nutriming solutions. Germination of somatic embryos nutrimed in nutriming solutions with pHs in the range of 5.5 - 6.8 prior to autoclaving, was comparable or improved above the the germination rates of the control un-nutrimed embryos (Table 3). However, the germination percentage of embryos nutrimed in solutions containing a starting pH 5 or less or 7 or greater was
- 25 adversely affected. The rates of shoot and root development were not affected by the pH or changes in the pH of the various nutriming solutions. Based on these results, it is evident that the pH of nutriming solutions can be in the range of 5.5 - 6.8.

**Table 3.** pH changes before and after autoclaving, after nutripriming and the effects on the growth and development of nutriprimed interior spruce somatic embryos.

PH treat-ments	Before auto-claving	After auto-claving	After nutri-priming	Germi-nation %	Shoot length (cm)	Root length (cm)
HRHT	-	-	-	89.0±6.2	1.29±0.03	0.86±0.04
5.0	5.0	4.62	4.77	75.8±9.6	1.05±0.03	0.93±0.03
5.5	5.5	5.17	4.78	95.0±1.3	1.21±0.03	0.87±0.04
5.8	5.8	5.35	4.81	83.0±7.1	1.08±0.03	0.94±0.04
6.0	6.0	5.61	4.89	97.5±1.1	1.22±0.03	0.95±0.05
6.2	6.2	5.81	5.04	95.1±2.2	1.08±0.03	0.97±0.03
6.5	6.5	6.13	5.63	93.3±3.1	1.10±0.03	1.00±0.03
6.8	6.8	6.43	5.89	90.8±3.7	1.10±0.03	0.86±0.03
7.0	7.0	6.57	6.14	73.3±12.2	1.00±0.07	0.91±0.04

5

#### EXAMPLE 4

##### Effects of nutripriming on *ex vitro* germination and rooting of pine and 10 spruce somatic embryos

Mature somatic embryos of interior spruce (*Picea glauca* (Moench) Voss x  
*P. engelmannii* Parry ex Engelm.), patula pine (*Pinus patula* Scheide et Deppe),  
radiata pine (*Pinus radiata* D. Don) and western white pine (*Pinus monticola*  
15 Dougl. ex D. Don) were desiccated with the HRHT treatment, and then  
nutriprimed in 3% w/v sucrose m24GMD solutions for 3 days under conditions as

previously described. The nutriprimed somatic embryos, along with un-nutriprimed controls, were then sown *ex vitro* and grown for three weeks. In the first study, the embryos were sown into soil-less mixes consisting of 50% screened peat and 50% fine perlite in 288-cell miniplug trays (Blackmore Co.); each cell contained a volume of 10 ml. In the second study, the embryos were sown into 400-cavity styrofoam miniplug trays filled with peat bound by a polymer (Grow-Tech Inc., San Bautista, CA). After sowing, the horticultural containers were placed into a non-sterile high-humidity ( $\geq 95\%$  RH) germination chamber for one week with an 18-hour photoperiod of  $30 - 40 \text{ mol m}^{-2}\text{s}^{-1}$  photosynthetic active radiation (PAR). In both studies, immediately after the trays containing *ex vitro* sown nutriprimed embryos were placed into the non-sterile high-humidity germination chamber, they were misted with m24GMD solution containing 3% w/v sucrose until the surfaces of the non-sterile growing substrates were well-wetted, and then at daily intervals for a period of 7 days. After the initial 7-day period in the non-sterile high-humidity germination environment, the trays were moved to a non-sterile misting chamber (RH ranged between 75-90%) for two more weeks of growth under an 18-hour photoperiod of  $120 - 150 \text{ mol m}^{-2}\text{s}^{-1}$  PAR with day-night temperatures of  $18-23^{\circ}\text{C}$ . Daily applications of the m24GMD nutrient solution containing 3% w/v sucrose were misted onto the germinants through to the end of the two-week period in the misting chamber. Aseptic techniques or conditions were not used for application of the nutrient solutions, or for the germination and growing environments during the 3-week culture period.

At the end of the three-week germination period, all germinants were counted and examined for root initiation. Germination and rooting percentages were calculated based on the total number of embryos sown in each replicate. The rooting percentage measures the plant conversion rate since germinants can have perfectly elongated and formed shoot, but without root initiation. The variability in replication and number of embryos in each replicates among the experiments was caused by embryo or space availability at the time of the experimentation.



The results of these experiments are summarized in Table 4. It was clear that both post-HRHT and nutriprimed embryos can be directly sown *ex vitro* and grown in conventional non-sterile nursery environments. However, it was clearly evident that nutripriming treatments prior to *ex vitro* sowing could significantly increase the germination percentage and also increase the plant conversion rate when compared to the control post-HRHT treatments which were not nutriprimed.

Table 4: Effects of nutripriming on *ex vitro* germination and rooting of spruce and pine somatic embryos

10

Species	Genotype	Cavities per plug tray	Embryo treatment	Germination %	% rooted
Interior spruce	4-2809	288*	Control	56.7	23.3
			Nutriprimed	75.6	62.4
<i>Pinus patula</i>	168-5074	288	Control	60.0	31.3
			Nutriprimed	88.1	54.2
	272-5071	288	Control	80.0	23.3
			Nutriprimed	100.0	45.6
	168-308	400**	Control	52.3	6.9
			Nutriprimed	73.3	30.0
<i>Pinus radiata</i>	B-7364	400	Control	51.4	5.6
			Nutriprimed	58.3	13.9

\* Volume of each cavity in a #288 plug tray is 10 ml

\*\* Volume of each cavity in a #400 plug tray is 3 ml

15

## EXAMPLE 5

**Ex vitro germination of nutrimed and desiccated somatic embryos of spruce and pine.**

5 Mature somatic embryos of interior spruce (*Picea glauca* (Moench) Voss x *P. engelmannii* Parry ex Engelm.), patula pine (*Pinus patula* Scheide et Deppe), and radiata pine (*Pinus radiata* D. Don) were desiccated with the HRHT treatment, and then nutrimed for 3 days in 3% (interior spruce) or 4% w/v  
10 sucrose m24GMD (patula and radiata pines) solutions under conditions as previously described. After removal from the nutriming solutions, the embryos were blotted dry on paper towels. The dried embryos were placed in Petri dishes. The dishes were then sealed in desiccation chambers containing unsaturated NaCl solutions, which provide varying relative humidity (RH) conditions as the  
15 molality of NaCl solutions changes. The embryos were desiccated in a 92.7% RH chamber at 23 °C in a Convion chamber for 24 hours. Then, the embryos were transferred to a 88.5% RH chamber and desiccated for another 48 hours at 5 °C in a refrigerator. Before and after each desiccation treatment, 3 samples of 3 embryos were taken from each genotype and determined for water content (WC)  
20 and relative water content (RWC). After desiccation, the embryos were rehydrated first in a 100% RH chamber containing water and then on agar overnight. After rehydration, the desiccated nutrimed embryos were sown into 400-cavity styrofoam miniplug trays filled with peat bound by a polymer (Grow-Tech Inc., San Bautista, CA). After sowing, the trays were placed into a non-  
25 sterile high-humidity ( $\geq 95\%$  RH) germination chamber for one week. In both studies, immediately after the trays containing *ex vitro* sown nutrimed embryos were placed into the non-sterile high-humidity germination chamber, they were misted with m24GMD solution containing 3% w/v sucrose until the surfaces of the non-sterile growing substrates were well-wetted, and then at daily intervals for a  
30 period of 7 days. After the initial 7-day period in the non-sterile high-humidity

germination environment, the trays were moved to a non-sterile misting chamber (RH ranged between 75-90%) for two more weeks of growth under an 18-hour photoperiod of 120 – 150 mol m<sup>-2</sup>s<sup>-1</sup> PAR with day-night temperatures of 18-23°C. Daily applications of the m24GMD nutrient solution containing 3% w/v sucrose  
5 were misted onto the germinants through to the end of the two-week period in the misting chamber. Aseptic techniques or conditions were not used for application of the nutrient solutions, or for the germination and growing environments during the 3-week culture period.

At the end of the three-week germination period, all germinated embryos  
10 and seedlings were counted and examined for root initiation. Germination and rooting percentages were calculated based on total number of embryos sown in each replicate. The rooting percentage measures the plant conversion rate since the survived germinants can have perfectly elongated and formed shoot, but without root initiation.

15 The results of the experiment are summarized in Table 5. From these results, it is clear that nutriprimed somatic embryos can be desiccated, then sown *ex vitro* into non-sterile horticultural growing media and successfully germinated.

Table 5: Germination and rooting percentage of nutriprimed somatic embryos which were desiccated prior to *ex vitro* sowing and germination

Species	Genotype	WC (%)		RWC (%)		Germination (%)	Rooting percentage (%)
		Before desiccation	After desiccation	Before desiccation	After desiccation		
Interior spruce	4-2809	230.5±12.6	59.7±4.9	61.6±2.3	20.1±1.5	52.6±3.5	39.7±6.9
	23-2672	235.7±8.6	72.8±3.9	75.9±3.9	24.9±2.4	68.3±4.2	54.4±4.8
Patula pine	168-308	266.9±25.6	119.4±18.2	84.8±5.7	42.1±3.6	74.4±6.1	7.78±6.2
Radiata pine	20-6609	270.9±39.0	100.1±12.8	75.1±5.7	33.4±3.6	88.8±3.8	35.0±5.0

#### EXAMPLE 6

#### 10 Effects of nutripriming on the *ex vitro* germination of spruce and pine somatic embryos in two different types of non-sterile horticultural growing substrates.

Mature somatic embryos of interior spruce (*Picea glauca* (Moench) Voss x  
 15 *P. engelmannii* Parry ex Engelm.), patula pine (*Pinus patula* Scheide et Deppe),  
 radiata pine (*Pinus radiata* D. Don), and , and western white pine (*Pinus*  
*monticola* Dougl. ex D. Don) were desiccated with the HRHT treatment, and then  
 nutriprimed in 3% w/v sucrose m24GMD solutions for 3 days under conditions as  
 previously described. Interior spruce line 4-2809 and *Pinus patula* lines 168-5074  
 20 and 2712-5071 nutriprimed somatic embryos, along with their un-nutriprimed  
 HRHT controls, were then sown *ex vitro* into 288-cell miniplug trays (Blackmore  
 Co.) containing a non-sterile horticultural growing mix comprised of 50%  
 screened peat and 50% fine perlite. *Pinus patula* line 168-308, *Pinus radiata* line  
 B-7364 and *Pinus monticola* line 212M-4572 were sown *ex vitro* into 400 cavity

styrofoam miniplug trays filled with peat bound by a polymer (Grow-Tech Inc., San Bautista, CA). After sowing, the containers were placed into a non-sterile growth chamber for one week under 18 hour photoperiod of 30 –40  $\mu\text{mol m}^{-2}\text{s}^{-1}$  PAR. In both studies, immediately after the trays containing *ex vitro* sown  
5 nutrimprimed embryos were placed into the non-sterile high-humidity germination chamber, they were misted with m24GMD solution containing 3% w/v sucrose until the surfaces of the non-sterile growing substrates were well-wetted, and then at daily intervals for a period of 7 days. After the initial 7-day period in the non-sterile high-humidity germination environment, the trays were moved to a  
10 non-sterile misting chamber (RH ranged between 75-90%) for two more weeks of growth under an 18-hour photoperiod of 120 – 150  $\text{mol m}^{-2}\text{s}^{-1}$  PAR with day-night temperatures of 18-23°C. Daily applications of the m24GMD nutrient solution containing 3% w/v sucrose were misted onto the germinants through to the end of the two-week period in the misting chamber. Aseptic techniques or conditions  
15 were not used for application of the nutrient solutions, nor for the germination and growing environments during the 3-week culture period.

At the end of the three-week germination period, all germinants were counted and examined for root initiation. Germination and rooting percentages were calculated based on the total number of embryos sown in each replicate.  
20 The rooting percentage measures the plant conversion rate since the germinants can have perfectly elongated and formed shoot, but no root initiation

The results for this study are summarized in Table 6. It was clearly evident that nutrimpriming somatic embryos prior to *ex vitro* sowing significantly improved germination percentage and increased the plant conversion rate when  
25 compared to the HRHT controls.

**Table 6:** Germination and rooting percentages of *ex vitro* sown HRHT control and nutriprimed somatic embryos of spruce and pine species.

Species	Genotype	Embryo treatment	Miniplug tray type	Germination %	Rooting %
Interior spruce	4-2809	HRHT control	288 cell	56.7	23.3
		Nutriprimed	288 cell	75.6	62.4
<i>Pinus patula</i>	168-5074	HRHT control	288 cell	60.0	31.3
		Nutriprimed	288 cell	88.1	54.2
<i>Pinus patula</i>	272-5071	HRHT control	288 cell	80.0	23.3
		Nutriprimed	288 cell	100	45.6
<i>Pinus patula</i>	168-308	HRHT control	400 cell	52.3	6.9
		Nutriprimed	400 cell	73.3	30.0
<i>Pinus radiata</i>	B-7364	HRHT control	400 cell	51.4	5.6
		Nutriprimed	400 cell	58.3	13.9
<i>Pinus monticola</i>	22M-4572	HRHT control	400 cell	80.0	41.2
		Nutriprimed	400 cell	56.7	50.0

**EXAMPLE 7****Effects of Ascorbic acid on germination and development of nutrimediated interior spruce somatic embryos**

5

Mature somatic embryos of interior spruce (*Picea glauca* (Moench) Voss x *P. engelmannii* Parry ex Engelm.) genotype 4-2809 were desiccated with the HRHT treatment, and then nutrimediated in 3% w/v sucrose m24GMD solutions for 3 days as previously described. The nutrimediation solutions were formulated with one of the following three ascorbic acid treatments; 0, 10  $\mu$ M, 100  $\mu$ M.

The nutrimediated somatic embryos were then sown into germination boxes containing 150 ml vermiculite plus 120 ml of 3% w/v sucrose m24GMD liquid medium. The liquid medium added to the germination boxes contained one of four ascorbic acid levels; 0, 10  $\mu$ M, 100  $\mu$ M or 1000 $\mu$ M. Nutrimediated embryos from each ascorbic acid nutrimediation treatment were then sown into triplicate boxes of each ascorbic acid-amended germination medium. After sowing, the germination boxes were sealed twice with Parafilm™. Embryos were germinated and grown for two weeks under 50-70  $\mu$ mol m<sup>-2</sup>s<sup>-1</sup> photosynthetic photon flux of 16-hour photoperiod at an air temperature of 22°C. Then, the germinants were harvested, and measured.

The effects of adding ascorbic acid on germination and rooting of nutrimediated interior spruce somatic embryo genotype 4-2809 are presented in Table 7. In this study the HRHT control somatic embryos germinated at very high percentages and consequently, improvements due to AA additions to the nutrimediation and germination media were not significant. However, the rooting percentage was significantly increased in the somatic embryos which received nutrimediation treatments containing ascorbic acid. Furthermore, it is evident that in this study, the largest rooting response was observed with embryos nutrimediated in solutions containing 10  $\mu$ M ascorbic acid.

30

Table 7: Germination and growth of interior spruce genotype 4-2809 somatic embryos nutriprimed in solutions containing ascorbic acid (AA) and germinated on vermiculite media containing AA.

AA level in germination media ( $\mu\text{M}$ )	AA level in priming solution ( $\mu\text{M}$ )	Germination %	Rooting %	Shoot length (cm)	Root length (cm)
0	HRHT	100.0 $\pm$ 0.0	86.7 $\pm$ 8.8	2.17 $\pm$ 0.08	3.14 $\pm$ 0.38
	0	93.3 $\pm$ 3.3	86.7 $\pm$ 3.3	2.07 $\pm$ 0.11	2.60 $\pm$ 0.37
	10	100.0 $\pm$ 0.0	86.7 $\pm$ 3.3	1.86 $\pm$ 0.10	2.59 $\pm$ 0.26
	100	100.0 $\pm$ 0.0	96.7 $\pm$ 3.3	2.07 $\pm$ 0.11	3.56 $\pm$ 0.37
1	HRHT	100.0 $\pm$ 0.0	75.6 $\pm$ 7.3	1.94 $\pm$ 0.09	2.44 $\pm$ 0.20
	0	96.7 $\pm$ 3.3	85.8 $\pm$ 3.0	1.68 $\pm$ 0.10	1.90 $\pm$ 0.23
	10	100.0 $\pm$ 0.0	86.7 $\pm$ 8.8	1.83 $\pm$ 0.11	2.07 $\pm$ 0.22
	100	100.0 $\pm$ 0.0	90.0 $\pm$ 0.0	1.79 $\pm$ 0.09	2.39 $\pm$ 0.28
10	HRHT	100.0 $\pm$ 0.0	75.0 $\pm$ 5.0	2.32 $\pm$ 0.15	3.70 $\pm$ 0.50
	0	97.5 $\pm$ 2.5	87.5 $\pm$ 6.3	1.84 $\pm$ 0.09	2.46 $\pm$ 0.30
	10	96.7 $\pm$ 3.3	86.7 $\pm$ 3.3	1.97 $\pm$ 0.19	2.82 $\pm$ 0.36
	100	100.0 $\pm$ 0.0	96.7 $\pm$ 3.3	2.33 $\pm$ 0.11	3.24 $\pm$ 0.32
100	HRHT	93.3 $\pm$ 6.7	68.5 $\pm$ 11.5	2.04 $\pm$ 0.12	3.51 $\pm$ 0.49
	0	93.3 $\pm$ 3.3	63.3 $\pm$ 8.8	1.81 $\pm$ 0.09	2.50 $\pm$ 0.33
	10	100.0 $\pm$ 0.0	78.2 $\pm$ 11.8	2.02 $\pm$ 0.11	3.18 $\pm$ 0.42
	100	100.0 $\pm$ 0.0	73.0 $\pm$ 11.8	2.12 $\pm$ 0.11	3.61 $\pm$ 0.39
1000	HRHT	100.0 $\pm$ 0.0	80.0 $\pm$ 5.8	2.05 $\pm$ 0.08	2.46 $\pm$ 0.30
	0	100.0 $\pm$ 0.0	90.0 $\pm$ 0.0	1.79 $\pm$ 0.14	2.11 $\pm$ 0.25
	10	100.0 $\pm$ 0.0	95.0 $\pm$ 5.0	2.00 $\pm$ 0.09	2.55 $\pm$ 0.20
	100	100.0 $\pm$ 0.0	90.0 $\pm$ 5.8	1.75 $\pm$ 0.15	2.19 $\pm$ 0.26



**EXAMPLE 8****Effects of growth regulators on germination and development of  
nutriprimed somatic embryos**

5

Mature somatic embryos of interior spruce (*Picea glauca* (Moench) Voss x *P. engelmannii* Parry ex Engelm.) genotypes 4-2809, 23-2672, and I-1026 were desiccated with the HRHT treatment. Genotype I-1026 was further desiccated at 85% RH for three days, then stored at  $-20^{\circ}\text{C}$ . Prior to nutripriming, genotype I-1026 was imbibed with a matipriming step which consisted of placing the desiccated embryos onto a screen, and then placing the screen on the surface of an agar substrate gelled with 3% phytoigel for 18 hrs. After this imbibition step was completed for genotype I-1026, all three genotypes were nutriprimed for 84 hr in 3% w/v sucrose m24GMD nutripriming solutions amended with the plant growth regulators described in Table 8.1.

Table 8.1: Plant growth regulator amendments added to 3% w/v sucrose m24GMD nutripriming solutions

Treatment #	Growth regulators added to nutripriming solutions		
	IBA ( $\mu\text{M}$ )	BA ( $\mu\text{M}$ )	GA <sub>477</sub> ( $\mu\text{M}$ )
1	0	0	0
2	0.1	0	0
3	0.05	0	0
4	0.05	0.01	0
5	0.05	0.01	0.01
6	0.05	0	0.05
7	HRHT control		

20

After the nutripriming step was completed, the nutriprimed somatic embryos were sown *ex vitro* into 400-cavity styrofoam miniplug trays filled with peat bound by a polymer (Grow-Tech Inc., San Bautista, CA). After sowing, the trays were placed into a non-sterile high-humidity ( $\geq 95\%$  RH) germination chamber for one week. In both studies, immediately after the trays containing *ex vitro* sown nutriprimed embryos were placed into the non-sterile high-humidity

germination chamber, they were misted with m24GMD solution containing 3% w/v sucrose until the surfaces of the non-sterile growing substrates were well-wetted, and subsequently at daily intervals for a period of 7 days. After the initial 7-day period in the non-sterile high-humidity germination environment, the trays were moved to a non-sterile misting chamber (RH ranged between 75-90%) for two more weeks of growth under an 18-hour photoperiod of 120 – 150 mol m<sup>-2</sup>s<sup>-1</sup> PAR with day-night temperatures of 18-23°C. Daily applications of the m24GMD nutrient solution containing 3% w/v sucrose were misted onto the germinants through to the end of the two-week period in the misting chamber. Aseptic techniques or conditions were not used for application of the nutrient solutions, or for the germination and growing environments during the 3-week culture period.

At the end of the three-week germination period, all germinated embryos and seedlings were counted and examined for root initiation. Germination and rooting percentages were calculated based on total number of embryos sown in each replicate. The rooting percentage measures the plant conversion rate since the survived germinants can have perfectly elongated and formed shoot, but without root initiation.

The results for this study are recorded in Table 8.2. The data indicate that for interior spruce genotype 4-2809, nutriming significantly increased both germination and rooting percentages compared to the HRHT control treatment. Addition of supplemental growth regulators to the nutriming solutions did not enhance germination and rooting performance of interior spruce genotype 4-2809 somatic embryos. However, a combination of 0.05 uM IBA plus 0.01 uM BA to solutions used to nutrimine genotype 23-2672 somatic embryos provided the highest germination response while the combinations of 0.05 uM IBA plus 0.01 uM BA, and combinations of 0.05 uM IBA plus 0.01 uM GA<sub>4/7</sub> provided the highest rooting percentage. Addition of 0.05 uM IBA to solutions used to nutrimine interior spruce genotype I-1026 provided the maximal germination and rooting response.

Table 8.2: Effects of plant growth regulators added to nutriming solutions on *ex vitro* germination and development of nutrimed interior spruce somatic embryos.

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Genotype	Dessication treatment	Nutriming treatment	Growth regulator level in nutriming solutions			Germination %	Rooting %
			IBA ( $\mu\text{M}$ )	BA ( $\mu\text{M}$ )	GA <sub>4/7</sub> ( $\mu\text{M}$ )		
4-2809	HRHT	Control	0	0	0	32.5 $\pm$ 2.5	7.5 $\pm$ 2.5
4-2809	HRHT	Primed	0	0	0	96.3 $\pm$ 2.4	81.3 $\pm$ 3.1
4-2809	HRHT	Primed	0.1	0	0	68.8 $\pm$ 5.2	51.3 $\pm$ 4.3
4-2809	HRHT	Primed	0.05	0	0	92.5 $\pm$ 6.0	82.5 $\pm$ 6.0
4-2809	HRHT	Primed	0.05	0.01	0	80.0 $\pm$ 3.5	61.3 $\pm$ 4.7
4-2809	HRHT	Primed	0.05	0.01	0.01	85.0 $\pm$ 4.6	77.5 $\pm$ 3.2
4-2809	HRHT	Primed	0.05	0	0.05	75.0 $\pm$ 2.0	65.0 $\pm$ 7.4
23-2672	HRHT	Control	0	0	0	57.5 $\pm$ 7.5	40.0 $\pm$ 10.0
23-2672	HRHT	Primed	0	0	0	76.7 $\pm$ 2.4	64.8 $\pm$ 7.9
23-2672	HRHT	Primed	0.1	0	0	82.5 $\pm$ 9.7	72.5 $\pm$ 6.3
23-2672	HRHT	Primed	0.05	0	0	90.0 $\pm$ 3.5	71.3 $\pm$ 6.6
23-2672	HRHT	Primed	0.05	0.01	0	95.0 $\pm$ 2.9	75.0 $\pm$ 5.4
23-2672	HRHT	Primed	0.05	0.01	0.01	85.0 $\pm$ 3.5	72.5 $\pm$ 4.3
23-2672	HRHT	Primed	0.05	0	0.05	81.3 $\pm$ 7.5	75.0 $\pm$ 7.4
I-1026	85% & frozen	Control	0	0	0	87.5 $\pm$ 2.5	47.5 $\pm$ 7.5
I-1026	85% & frozen	Primed	0	0	0	78.8 $\pm$ 3.8	68.8 $\pm$ 5.2
I-1026	85% & frozen	Primed	0.1	0	0	88.8 $\pm$ 6.6	58.8 $\pm$ 5.9
I-1026	85% & frozen	Primed	0.05	0	0	93.8 $\pm$ 2.4	66.3 $\pm$ 4.7
I-1026	85% & frozen	Primed	0.05	0.01	0	77.5 $\pm$ 6.3	30.0 $\pm$ 7.4
I-1026	85% & frozen	Primed	0.05	0.01	0.01	73.8 $\pm$ 8.8	31.3 $\pm$ 2.4
I-1026	85% & frozen	Primed	0.05	0	0.05	63.8 $\pm$ 6.9	45.0 $\pm$ 5.8

The second study assessing the effects of plant growth regulators on germination performance of nutrimed somatic embryos involved additions of plant growth regulators to the horticultural growing substrates after the nutrimed somatic embryos were sown *ex vitro*. In this study, somatic embryos from interior spruce genotypes 4-2809, 23-2672, and I-1026 were desiccated and stored as described for the first growth regulator study. Prior to nutriming, genotype I-1026 was imbibed with a matriming step which consisted of placing the desiccated embryos onto a screen, and then placing the screen on the surface of an agar substrate gelled with 3% phytozel for 18 hrs. After this imbibition step was completed for genotype I-1026, all three genotypes were nutrimed for 84 hr in 3% w/v sucrose m24GMD nutriming solutions and then sown *ex vitro* into 400-cavity styrofoam miniplug trays filled with peat bound by a polymer (Grow-Tech Inc., San Bautista, CA). After sowing was completed, the nutrimed embryo received exogenous applications of one of the treatments described in Table 8.3.

Table 8.3: Plant growth treatments added to horticultural substrates containing *ex vitro*-sown nutrimed somatic embryos.

Treatment #	Growth regulators applied to <i>ex vitro</i> -sown nutrimed embryos		
	IBA ( $\mu$ M)	BA ( $\mu$ M)	GA <sub>4/7</sub> ( $\mu$ M)
1	0	0	0
2	0.2	0	0
3	0.1	0	0
4	0.1	0.02	0
5	0.1	0.02	0.02
6	0.1	0	0.1
7	HRHT control		

After the exogenous application of the plant growth regulator treatments, the blocks were placed into a non-sterile high-humidity ( $\geq 95\%$  RH) germination chamber for one week. Immediately after the trays containing *ex vitro* sown nutrimed embryos were placed into the non-sterile high-humidity germination chamber, they were misted with m24GMD solution containing 3% w/v sucrose

until the surfaces of the non-sterile growing substrates were well-wetted, and then at daily intervals for a period of 7 days. After the initial 7-day period in the non-sterile high-humidity germination environment, the trays were moved to a non-sterile misting chamber (RH ranged between 75-90%) for two more weeks of growth under an 18-hour photoperiod of  $120 - 150 \text{ mol m}^{-2}\text{s}^{-1}$  PAR with day-night temperatures of 18-23°C. Daily applications of the m24GMD nutrient solution containing 3% w/v sucrose were misted onto the germinants through to the end of the two-week period in the misting chamber. Aseptic techniques or conditions were not used for application of the nutrient solutions, nor for the germination and growing environments during the 3-week culture period.

At the end of the three-week germination period, all survived germinants were counted and examined for root initiation. Germination and rooting percentages were calculated based on total number of embryos sown in each replicate.

The results for this study are recorded in Table 8.4. As in the previous study, exogenous applications of plant growth regulators to nutriprimed interior spruce somatic embryos from genotype 4-2809 did not improve germination or rooting performance. However, exogenous application of IBA alone or in combination with 0.02  $\mu\text{M}$  Ba significantly enhanced germination performance.

Table 8.4: Effects of plant growth regulators added to the horticultural growing substrate on *ex vitro* germination and development of nutrimed interior spruce somatic embryos.

Genotype	Dessication treatment	Nutriming treatment	Growth regulator level in growing substrate			Germination %	Rooting %
			IBA ( $\mu$ M)	BA ( $\mu$ M)	GA <sub>4/7</sub> ( $\mu$ M)		
4-2809	HRHT	Control	0	0	0	32.5 $\pm$ 2.5	7.5 $\pm$ 2.5
4-2809	HRHT	Primed	0	0	0	96.3 $\pm$ 2.4	81.3 $\pm$ 3.1
4-2809	HRHT	Primed	0.2	0	0	97.5 $\pm$ 1.4	78.8 $\pm$ 7.5
4-2809	HRHT	Primed	0.1	0	0	92.5 $\pm$ 7.5	75.0 $\pm$ 10.
4-2809	HRHT	Primed	0.1	0.02	0	95.0 $\pm$ 3.5	73.8 $\pm$ 3.1
4-2809	HRHT	Primed	0.1	0.02	0.2	90.0 $\pm$ 5.4	72.5 $\pm$ 8.5
4-2809	HRHT	Primed	0.1	0	0.1	83.8 $\pm$ 2.4	61.3 $\pm$ 10.
23-2672	HRHT	Control	0	0	0	57.5 $\pm$ 7.5	40.0 $\pm$ 10.
23-2672	HRHT	Primed	0	0	0	76.7 $\pm$ 2.4	64.8 $\pm$ 7.9
23-2672	HRHT	Primed	0.2	0	0	72.5 $\pm$ 10.9	67.5 $\pm$ 10.
23-2672	HRHT	Primed	0.1	0	0	72.5 $\pm$ 11.	61.3 $\pm$ 13.
23-2672	HRHT	Primed	0.1	0.02	0	74.8 $\pm$ 5.5	64.7 $\pm$ 4.8
23-2672	HRHT	Primed	0.1	0.02	0.2	76.3 $\pm$ 9.4	68.8 $\pm$ 8.0
23-2672	HRHT	Primed	0.1	0	0.1	58.3 $\pm$ 8.5	48.8 $\pm$ 5.2
I-1026	85% & frozen	Control	0	0	0	87.5 $\pm$ 2.5	47.5 $\pm$ 7.5
I-1026	85% & frozen	Primed	0	0	0	78.8 $\pm$ 3.8	68.8 $\pm$ 5.2
I-1026	85% & frozen	Primed	0.2	0	0	80.0 $\pm$ 6.8	65.0 $\pm$ 5.4
I-1026	85% & frozen	Primed	0.1	0	0	91.3 $\pm$ 5.5	66.3 $\pm$ 3.7
I-1026	85% & frozen	Primed	0.1	0.02	0	83.8 $\pm$ 5.5	71.3 $\pm$ 5.5
I-1026	85% & frozen	Primed	0.1	0.02	0.2	82.5 $\pm$ 2.5	63.8 $\pm$ 4.7
I-1026	85% & frozen	Primed	0.1	0	0.1	82.5 $\pm$ 6.0	67.5 $\pm$ 4.3

5

These examples demonstrate that additions of selected plant growth regulators to nutriming solutions or alternatively, exogenous applications to *ex vitro*-sown nutrimed somatic embryos can significantly improve the germination and rooting of somatic embryos. Those skilled in the art will be able to use this information disclosed herein to determine which plant growth regulators, alone or

10

in combination, and at what rates, can be used to enhance the *ex vitro* germination and development of somatic embryos of their preferred species.

### Example 9

- 5        The objectives for these studies were to determine the effects of environmental conditions during the nutrimpriming step on the subsequent *ex vitro* germination and growth of nutrimprimed somatic embryos.

      The first study assessed the effects of light and temperature conditions during nutrimpriming on subsequent germination and development performance.

10    Mature somatic embryos of interior spruce (*Picea glauca* (Moench) Voss x *P. engelmannii* Parry ex Engelm.) genotype 23-2672 were desiccated with the HRHT treatment, and then divided into five groups of 160 somatic embryos per group. One group was not primed and served as the HRHT control treatment for this study. The remaining four groups were randomly assigned for nutrimpriming in

15    250-ml flasks containing 3% w/v sucrose m24GMD nutrient solutions. The nutrimpriming step was performed at 5°C temperature with or without light, or at ambient temperature (i.e., 20-23°C) conditions with or without light. When light was applied during the nutrimpriming process, the photoperiod was 24 hours of 30  $\mu\text{mol m}^{-2}\text{s}^{-1}$  photosynthetic photon flux. The dark treatment was done by wrapping

20    the flasks with aluminum foil. The duration of each nutrimpriming treatment was 72 hours.

      After the nutrimpriming step was completed, each treatment was divided into eight groups of twenty embryos and sown by hand on vermiculite media in Sigma polycarbonate boxes. The liquid media in these germination boxes were

25    m24GMD with or without 3% w/v sucrose. Four of the eight groups were sown on media without sucrose while the remaining four groups were sown on media containing 3% w/v sucrose. The experiment design was a random complete block design for each media type. The germination boxes were placed in a germination room and grown for two weeks under 50-70  $\mu\text{mol m}^{-2}\text{s}^{-1}$

30    photosynthetic photon flux of 16-hour photoperiod at an air temperature of 23°C.

The germinants were then harvested and assessed for germination rates, hyperhydricity rates, shoot and root length. Germination was defined as germinants having a root longer than 2 mm, and elongated hypocotyl. Hyperhydricity was defined by the thickening of hypocotyl and watery appearance of the germinants, whether germinated or not germinated. The results for this study are summarized in Tables 9.1 and 9.2. Temperatures at which nutriming was performed did not affect the subsequent germination and development of nutrimed somatic if 3% w/v sucrose was added to the nutriming solutions. However, in the absence of 3% w/v sucrose in the nutriming solutions, only those embryos which were nutrimed at ambient temperatures germinated successfully. However, the root development of somatic embryos nutrimed in solutions without sucrose, was significantly reduced when compared to those nutrimed in solutions containing sucrose. In conclusion, nutriming can be performed under refrigerated conditions (e.g., 4°C) if so desired, if sucrose is included as a component of the nutriming solution.

**Table 9.1:** Effects of temperature and light during nutriming on germination of interior spruce somatic embryos.

Nutriming treatment		Germination media			
		m24GMD plus 3% sucrose		m24GMD	
		Germination %	% with hyperhydricity	Germination %	% with hyperhydricity
5 °C	Dark	100	0	0	46.3±26.9
	Light	100	2.4±1.4	3.8±2.4	47.5±27.5
20-23°C	Dark	100	2.5±2.5	100	42.5±24.6
	Light	100	2.4±1.4	91.7±8.3	40.0±23.2
HRHT control		93.8±4.7	0	25.0±2.5	10.0±4.1



**Table 9.2.** Effects of temperature and light during nutripriming on growth and development of interior spruce somatic embryos.

Nutripriming treatment		Germination media			
		m24GMD plus 3% sucrose		m24GMD	
		Shoot length (mm)	Root length (mm)	Shoot length (mm)	Root length (mm)
5°C	Dark	15.9±0.4	12.5±0.8	0	0
	Light	15.8±0.4	12.9±0.7	11.4±0.3	1.5±0.1
20-23°C	Dark	17.4±0.4	12.4±0.8	12.7±0.3	2.9±0.1
	Light	18.1±0.4	11.6±0.9	14.7±0.3	2.4±0.1
HRHT control		15.3±0.4	11.2±0.7	10.9±0.5	0.2±0.1

- 5 The second study assessed the effects adding various osmotica to nutripriming solutions on subsequent germination and development performance. Mature somatic embryos of interior spruce (*Picea glauca* (Moench) Voss x *P. engelmannii* Parry ex Engelm.) genotype 23-2672 were desiccated with the HRHT treatment, and then divided into 10 groups of 160 somatic embryos per
- 10 group. One group was not primed and served as the HRHT control treatment for this study. The remaining nine groups were randomly assigned to one of the following osmoticant treatments added to the m24GMD nutripriming solution: 1% w/v betaine, 1.65% w/v fructose, 1.65% w/v glucose, 1.65% w/v mannitol, 1.65% w/v sorbitol, 3% w/v lactose, 3% w/v maltose, 3% w/v sucrose or 10% w/v
- 15 polyethylene glycol (PEG) 4000. The final osmotic potential in all treatments was of -0.35 MPa except for the PEG solution, which had an osmotic potential of -0.32 MPa. The nutripriming solutions and embryos were placed into 250-ml Kimax® baffled flasks. The nutripriming duration was 72 hrs at temperatures ranging between 20-23°C, and a 24-hr photoperiod of 30  $\mu\text{mol m}^{-2}\text{s}^{-1}$
- 20 photosynthetic photon flux.

At the completion of the nutriming step, the nutrimed embryos from each treatment were divided into 8 groups of 20 embryos and sown by hand onto vermiculite media in Sigma polycarbonate boxes. The liquid media in these germination boxes were either 3% w/v sucrose m24GMD or m24GMD without 5 sucrose. The experiment design was a random complete block design for each media type. Each nutriming treatment had 4 replicates of 20 embryos in each germination media type. The germination boxes were placed in a germination room and grown for two weeks under 50-70  $\mu\text{mol m}^{-2}\text{s}^{-1}$  photosynthetic photon flux of 16-hour photoperiod at an air temperature of 23°C. The germinants were 10 then harvested and assessed for germination rates, hyperhydricity rates, shoot and root length. Germination was defined as germinants having a root longer than 2 mm, and elongated hypocotyl. Hyperhydricity was defined by the thickening of hypocotyl and watery appearance of the germinants, whether germinated or not germinated. The results for this study are summarized in 15 Tables 9.3 and 9.4.

In summary, although addition of osmoticants to nutriming solutions containing 3% w/v sucrose did not adversely affect germination percentage, all osmoticant treatments with the exception of betain, mannitol and lactose caused increased levels of hyperhydricity. When the osmoticants were added to 20 nutriming solutions which did not contain sucrose, germination performance became variable and high levels of hyperhydricity were observed (Table 9.3). Nutrimed embryos from all osmoticant treatments formed roots and shoots, but the best performance was observed in embryos nutrimed in 3% w/v sucrose m24GMD nutriming solution that did not contain any osmoticants (Table 9.4).

**Table 9.3:** Effects of osmoticants on germination of nutrimed interior spruce somatic embryos.

Osmoticant added to nutriming solution	Germination media			
	m24GMD plus 3% sucrose		m24GMD	
	Germination %	% with hyperhydricity	Germination %	% with hyperhydricity
1% betaine	100	2.5±2.5	45.0±26.3	76.3±19.1
1.65% fructose	100	16.3±9.4	87.5±7.8	86.3±9.4
1.65% glucose	96.3±2.4	18.2±6.9	96.2±2.4	58.8±8.0
1.65% mannitol	100	4.7±3.2	24.6±12.7	93.5±5.0
1.65% sorbitol	100	7.5±2.5	5.0±5.0	92.6±6.0
3% lactose	100	3.7±2.4	27.6±24.3	100
3% maltose	98.8±1.3	17.5±7.8	0	100
3% sucrose	100	2.4±1.4	91.7±8.3	40.0±23.2
10% PEG 4000	100	6.0±6.0	6.3±6.3	97.5±4.3
HRHT control	93.8±4.7	0	25.0±25.0	10.0±4.1

**Table 9.4:** Effects of osmoticants on the development of nutriprimed interior spruce somatic germinants.

Osmoticant added to nutripriming solution	Germination media			
	m24GMD plus 3% sucrose		m24GMD	
	Shoot length (mm)	Root length (mm)	Shoot length (mm)	Root length (mm)
1% betaine	14.2±0.3	12.2±0.8	11.2±0.3	0.4±0.2
1.65% fructose	13.5±0.3	12.2±0.8	10.0±0.3	2.1±0.1
1.65% glucose	14.8±0.4	10.8±0.8	10.8±0.3	1.7±0.2
1.65% mannitol	13.6±0.3	12.0±0.5	10.0±0.3	0.3±0.1
1.65% sorbitol	14.5±0.4	15.5±1.0	10.7±0.3	0.4±0.1
3% lactose	15.5±0.3	12.4±0.8	11.5±0.2	0.3±0.1
3% maltose	14.2±0.3	11.5±0.8	11.2±0.3	0.8±0.1
3% w/v sucrose	18.1±0.4	11.6±0.9	14.7±0.3	2.4±0.1
10% PEG 4000	14.8±0.3	12.2±1.1	10.8±0.3	0.3±0.1
HRHT control	13.2±0.5	12.6±1.2	10.9±0.5	0.2±0.1

5

**EXAMPLE 10**

The objective of this study was to assess the effects of nutripriming on the development of nutrient reserves within somatic embryos of interior spruce.

Mature somatic embryos of interior spruce (*Picea glauca* (Moench) Voss x *P. engelmannii* Parry ex Engelm.) genotype I-1026 were desiccated with the HRHT treatment.

Nine groups of 20 embryos were randomly picked up from the post-HRHT population. The length of each embryo was measured. Subsequently, four groups of embryos were nutriprimed in m24GMD solutions containing 2% w/v sucrose for durations of 1,2,3 or 4 days. The remaining 4 groups of embryos were nutriprimed in m24GMD solutions containing 4% w/v sucrose for durations

of 1,2,3 or 4 days. At the conclusion of each nutriming treatment, embryos were collected on 53- $\mu$  nylon mesh and rinsed three times with deionized water. The embryos were then blotted dry with filter paper and their post-nutriming lengths measured. Each group was then divided into 5 sets of 4 embryos and 5 were placed in tared microcentrifugal tubes. After fresh weight determinations, the embryos were dried at 60 °C for 24 hours and their dry weight determined. The ninth group, which consisted of non-nutrimed post-HRHT embryos (i.e., the controls), was also divided into 5 sets of 4 embryos for determinations of length, fresh and dry weights.

10 An additional 8 groups of 160 embryos per group were sorted from the the post-HRHT treated embryos. Four groups of embryos were nutrimed in m24GMD solutions containing 2% w/v sucrose for durations of 1,2,3 or 4 days. The remaining 4 groups of embryos were nutrimed in m24GMD solutions containing 4% w/v sucrose for durations of 1,2,3 or 4 days. At the conclusion of 15 each nutriming treatment, embryos were collected on 53- $\mu$  nylon mesh and rinsed three times with deionized water. The embryos were then blotted dry with filter paper. Each group was then randomly divided into 16 samples of 15 embryos. One sample was placed in a tared 1.5-ml microcentrifugal tube and weighed for fresh weight. The tubes were then dropped in liquid N<sub>2</sub> for 10 min 20 and stored at -80 °C until biochemical analyses could be done as outlined below.

#### ***Determination of Triacylglyceride contents***

The protocols for extraction, purification, and assay of triacylglyceride (TAG) followed the procedures of Feirer et al. (1989). Briefly, samples of (a) 15 nutrimed or, (b) 15 non-nutrimed somatic embryos, or (c) 10 zygotic 25 embryos which were dissected from dry seeds from each of three seedlots (SL#s 28840; 60245; 08729) were ground for 2 minutes in a 1.5-ml microcentrifugal tube with 50 mg of aluminum oxide (Sigma, Activity Grade I) and 0.1 ml of anhydrous isopropanol (Fisher, HPLC grade) using a motorized pestle. The pestle was washed three times with 0.3 ml of isopropanol. The washing solutions 30 were collected in the microcentrifugal tube. The tubes were then placed on a

shaker (150 rpm) for 15 minutes, followed by 15 minutes centrifugation at 13,000 rpm.

To purify the lipid extracts, a 0.8-ml aliquot of supernatant was withdrawn from each sample and added into a 10-ml test tube containing 0.8 g of aluminum oxide and 1.8 ml of isopropanol. The tubes were placed on a shaker at 150 rpm for 15 minutes and then centrifuged at 3,000 rpm for 20 minutes. The supernatant was transferred to a new tube. The pellet was resuspended in 2.6 ml of isopropanol. The tubes were again placed on the shaker, resuspended and re-centrifuged. The resultant supernatant was pooled with the initial supernatant collected from the sample. To quantify TAG levels in the purified extracts, an 1-ml aliquot of the pooled supernatant was withdrawn, placed in a 1.5 -l microcentrifuge tube, and recentrifuged at 13,000 rpm for 15 min. A 0.8-ml aliquot of supernatant was withdrawn and placed in a 5-ml polypropylene tube. A 0.2-ml aliquot of 1 N KOH was added to each tube. The tubes were capped, vortexed, and placed in a 60°C water bath for 5 minutes to enable the following reaction to occur:



After 5-minute reaction period, the tubes were removed from the water bath and cooled in a basin filled with running tap water for 2 minutes. Then, 0.2-ml aliquot of sodium m-periodate was added to each tube to facilitate the following reaction:



After a 10-min incubation, 1.2-ml aliquot of color reagent ( a mixture of ammonium acetate and acetylacetone in isopropanol) was added to each tube, after which, the tubes were placed in a 60°C water bath for 30 min to enable the following reaction to take place:



The tubes were then cooled in a basin filled with running tap water for 2 minutes. The absorbance in each solution was determined in a spectrophotometer set at 410 nm.

### Results

5 Nutripriming affected the onset of biological activity within somatic embryos as evidenced by elongation along their embryonyl axes (Table 10.1). Furthermore, as the duration of nutripriming treatments was increased, the magnitude of embryo elongation also increased (Table 10.1). In this study, embryo elongation was more pronounced when nutripriming solutions were  
10 amended with 2% w/v sucrose as compared to 4% w/v sucrose. Also, it appeared that the maximal response occurred with 3 - 4 day nutripriming treatments.

15 Table 10.1: Length (mm) of interior spruce genotype I-1026 somatic embryos before and after nutripriming treatments.

Duration of nutripriming (days)	Post-HRHT control	Sucrose level in nutripriming solution			
		2% in m24GMD		4% in m24GMD	
		Embryo length before nutripriming	Embryo length after nutripriming	Embryo length before nutripriming	Embryo length after nutripriming
0	2.6±0.1				
1		2.1±0.1	2.3±0.1	2.4±0.1	2.5±0.1
2		2.1±0.1	2.6±0.1	2.2±0.1	2.6±0.1
3		2.5±0.1	3.5±0.1	2.1±0.1	2.8±0.1
4		2.6±0.1	4.1±0.1	2.1±0.1	3.2±0.1

Furthermore, it was clearly apparent that increasing the duration of the nutriming periods resulted in significant increases in embryonal biomass formation as evidenced by increases in dry weights compared to the dry weight HRHT control embryos, of 32-54% after 3 days of nutriming, and 65 - 75% after 4 days of nutriming (Table 10.2).

Table 10.2. Effects of nutriming on dry weights (mg) of interior spruce genotype I-1026 somatic embryos ( means of 200 embryos).

Embryo treatment	Duration of nutriming (days)	Initial dry weight of embryos	Dry weight of embryos after nutriming in	
			2% w/v sucrose m24GMD	4% sucrose m24GMD
HRHT control	-	0.281±0.010		
Nutrimed	1		0.240±0.010	0.296±0.009
	2		0.314±0.011	0.342±0.013
	3		0.373±0.020	0.434±0.014
	4		0.464±0.027	0.491±0.014

10

The concentration of sucrose in nutriming solutions and the duration of nutriming significantly affected the accumulation of storage reserve lipids, i.e., TAGs, in interior spruce somatic embryos (Table 10.3). While TAG levels increased over 90% in embryos nutrimed for one day in solutions containing 2% w/v sucrose, the TAG levels subsequently declined as the duration nutriming was increased, to the point where by the fourth day of nutriming, the TAG levels were only 10% greater than in the non-nutrimed controls. On the other hand, the TAG levels in somatic embryos nutrimed in solutions containing 4% w/v sucrose doubled within the first day and remained at that level through the fourth day of nutriming. Furthermore, it should be noted that although the TAG levels in the HRHT control somatic embryos were comparable to those in zygotic embryos, embryos nutrimed in solutions containing 4% w/v sucrose contained 85% more TAG than were present in the zygotic embryos.



Table 10.3: Comparison of lipid levels in interior spruce zygotic embryos with control and nutriprimed somatic embryos of genotype I-1026

Embryo source	Nutripriming treatment	Priming duration (days)	Lipid content ( $\mu\text{g}/\text{embryo}$ )
Zygotic seed lot#			
28840			52.3 $\pm$ 5.8
60245			68.7 $\pm$ 2.9
08729			65.8 $\pm$ 4.3
Somatic genotype I-1026	HRHT control		55.6 $\pm$ 5.7
Somatic genotype I-1026	2% sucrose in m24GMD	1	107.6 $\pm$ 12.1
		2	112.2 $\pm$ 11.1
		3	64.1 $\pm$ 5.6
		4	63.0 $\pm$ 2.7
Somatic genotype I-1026	4% sucrose in m24GMD	1	113.9 $\pm$ 17.1
		2	112.9 $\pm$ 10.1
		3	113.8 $\pm$ 11.8
		4	113.2 $\pm$ 14.6

5        These results clearly demonstrate that nutripriming somatic embryos in solutions containing sucrose and mineral nutrients facilitates and enhances the physiological and biochemical processes associated with the post-imbibition events that occur in Phase 2 of germination. Furthermore, it is evident that nutripriming enables somatic embryos to accumulate storage reserve lipids which

10 enhance the later events associated with completion of germination and the onset of seedling growth and development.

**EXAMPLE 11**

The objective of this study was to examine the effects of nutrimpriming duration, post-nutrimpriming desiccation, and post-desiccation storage on *ex vitro* germination of interior spruce somatic embryos in non-sterile substrates and environments.

Mature somatic embryos of interior spruce (*Picea glauca* (Moench) Voss x *P. engelmannii* Parry ex Engelm.) genotypes 4-2809 and I-1026 were desiccated with the HRHT treatment and stored at 4°C. Embryos were nutrimprimed for one of 1, 2, or 3 days in ½ m24GMD liquid medium containing 3% w/v sucrose in 250-ml Kimax® baffled Erlenmeyer culture flasks as described in previous examples.

Embryos nutrimprimed from each treatment, along with post-HRHT controls, were divided into groups of 155 embryos. Each group was placed on an 800-µm nylon screen in a 6-cm Petri dish and randomly assigned to one of two desiccation treatments. Desiccation treatment #1 was 90.0% RH (embryos desiccated over a 2.83-mol NaCl solution) while desiccation treatment #2 was 85.0% RH (embryos desiccated over saturated KCl solutions). Both desiccation treatments were carried out in sealed chambers at 20°C for 3 days. Each "nutrimpriming period X desiccation" treatment combination for each genotype had 3 groups that were desiccated in three chambers. At the end of each desiccation treatment, 1 sample of 5 embryos was taken from each chamber for measurement of water content based on differences in fresh and oven-dry weights (3 days @ 65°C) weight. One group was immediately imbibed using a matrimpriming process on semi-solid substrate comprised of 0.6% agar, 3% w/v sucrose, and ½m24GMD germination medium in 10-cm Petri dishes for 68 hours at 23 °C in a Convion tissue culture chamber (Convion Ltd., Winnipeg, MA). The imbibed embryos were then sown either *in vitro* or *ex vitro* as described below.

The other two groups of embryos were scraped off the nylon screens into sterilized 2.0-ml polypropylene, low-temperature freezer vials (VWR Canlab, Mississauga, ON). The vials were capped. One group was stored at  $-20^{\circ}\text{C}$  in a freezer and the other group was stored at  $4.0^{\circ}\text{C}$ . After one week of storage, the  
5 desiccated nutriprimed embryos were imbibed with the matipriming process described previously, then germinated *in vitro* or *ex vitro*. At each sowing time, there were un-desiccated controls which received similar HRHT or priming treatments to the desiccated nutriprimed embryos.

*In vitro* germination was on the same medium as described for imbibition,  
10 i.e., on a semi-solid substrate comprised of 0.6% agar, 3% w/v sucrose, and  $\frac{1}{2}$ m24GMD germination medium in 10-cm Petri dishes. Each "genotype X nutripriming duration X desiccation" treatment combination had 3 replicates of 10 embryos in a 10-0cm Petri dish. Germination proceeded under sterile conditions in an environment-controlled tissue culture room.

15 *Ex vitro* germination was conducted in a peat-based polymer-bound soilless mix dispensed into 504-cell (4 ml/cell) Styrofoam miniplug trays (Grow-Tech Inc., San Bautista, CA). The *ex vitro* experiment had 3 replicates of 20 embryos for each treatment combination.

The sown trays were placed in a conventional non-sterile horticulture  
20 germination chamber in an environment-controlled growth room. The environmental conditions were  $22-24^{\circ}\text{C}$  air temperature, 80-100% RH, 0.10 - 0.15%  $\text{CO}_2$ , and a 16-hr photoperiod with  $40-120\ \mu\text{mol m}^{-2}\text{s}^{-1}$  photosynthetic photon flux. Immediately after the trays containing *ex vitro* sown nutriprimed embryos were placed into the non-sterile germination chamber, they were misted  
25 with a 3% w/v sucrose solution until the surfaces of the non-sterile growing substrates were well-wetted. Subsequently, the trays were misted with the 3% w/v sucrose solution daily for a 2-week germination period. Mineral nutrients in the form of a plant-starter fertilizer solution at a rate of  $1\text{g l}^{-1}$  of 11-41-8 N-P-K (Plant Products Co. Ltd., Brampton, ON) were misted onto the germinating  
30 embryos at 2-day intervals. Aseptic techniques or conditions were not used for application of the nutrient solutions, nor for the germination environment during the 2-week culture period. Furthermore, prophylactic applications of 0.1 g/l

benlate, 0.05g/l ampicillin plus 0.05g/l penicillin G (or 0.1 ampicillin), and 5 ml/l Gnatrol were applied at 5-day intervals to control fungi, bacteria, and fungus fly larvae.

The data in Table 11.1 indicate that a greater degree of desiccation occurred with nutriprimed embryos when they were desiccated in environments with RH values of 85% compared to desiccation environments with 90% RH.

**Table 11.1:** Water content (based on fresh and dry weight) of embryos at the end of desiccation treatment before being imbibed for germination or placed in low temperature storage.

Interior spruce genotype	Nutripriming Duration (days)	Desiccation treatment (% RH)	Water content (% fresh weight)	Water content (% dry weight)
4-2809	HRHT control	85	11.47±0.68	12.97±0.87
		90	11.44±1.55	12.98±1.98
	1	85	17.52±2.72	21.50±4.02
		90	22.10±1.73	28.50±2.82
	2	85	25.15±6.69	35.98±13.34
		90	20.61±1.73	26.08±2.81
	3	85	22.00±2.01	28.38±3.23
		90	31.59±2.17	46.50±4.80
I-1026	HRHT control	85	17.36±2.93	21.33±4.47
		90	19.75±1.54	24.71±2.36
	1	85	16.06±0.35	19.14±0.49
		90*	-	-
	2	85	20.36±0.46	25.57±0.73
		90	40.34±5.80	71.12±18.05
	3	85	22.49±0.51	29.03±0.85
		90	51.65±0.65	106.88±2.77

5 \* Salt solutions inadvertently saturated at least one of the three replicates in this treatment. Consequently, the results for this treatment were compromised.

The data in Table 11.2 demonstrate that somatic embryos can be desiccated and stored after nutripriming treatments are completed, without significant losses in germination vigour or subsequent seedling growth and development. The data also demonstrate that desiccated nutriprimed somatic embryos can be stored under a wide range of temperature conditions ranging from ambient (i.e., 23°C) to refrigerated (i.e., 4°C), and frozen storage (i.e., -20°C). We surprisingly found that embryos desiccated after 3 days of nutripriming in solutions containing 3% w/v sucrose, and stored at ambient temperatures maintained 100% germination

vigour and conversion into fully intact seedlings when sown *ex vitro* and germinated in non-sterile growing media cultured in non-sterile environmental conditions, while those stored at -20°C demonstrated 93% germination success and 80% conversion into fully intact seedlings when sown and germinated in the same non-sterile environments.

**Table 11.2:** Effects of desiccation treatment and storage conditions on subsequent germination and growth of nutrimed somatic embryos.

10

Duration of priming (days)	Desiccation treatment (% RH)	Storage Temp (°C)	Germination %	Rooting %
HRHT control	Control 1	23	100	100
	Control 2	23	100	96.7±3.3
	85	23	100	75.0±8.3
		4	100	100
		-20	100	100
	90	23	100	100
		4	100	100
		-20	70.0±20.0	55.0±5.0
1	Control 1	23	96.7±3.3	80.0±5.8
	Control 2	23	100	96.7±3.3
	85	23	96.7±3.3	66.7±14.5
		4	96.7±3.3	36.7±14.5
		-20	100	56.7±3.3
	90	23	100	96.7±3.3
		4	100	93.3±3.3
		-20	100	100.0±0.0
2	Control 1	23	100	93.3±3.3
	Control 2	23	100	100.0±0.0
	85	23	96.7±3.3	86.7±8.8
		4	96.7±3.3	60.0±10.0
		-20	100	76.7±6.7
	90	23	100	96.7±3.3
		4	96.7±3.3	70.0±5.8
		-20	100	93.3±6.7

Duration of priming (days)	Desiccation treatment (% RH)	Storage Temp (°C)	Germination %	Rooting %
3	Control 1	23	100	100
	Control 2	23	100	100
	85	23	96.7±3.3	73.3±8.8
		4	76.7±18.6	33.3±8.8
		-20	95.0±5.0	55.0±35.0
	90	23	100	100
		4	46.7±3.3	33.3±6.7
		-20	93.3±6.7	80.0±5.8

### EXAMPLE 12

- 5        The objective for this study was to assess the effects of : (a) elevated sucrose levels in nutrimpriming solutions, and (b) extended nutrimpriming durations on the germination performance os somatic embryos.

Mature somatic embryos of interior spruce (*Picea glauca* (Moench) Voss x *P. engelmannii* Parry ex Engelm.) genotypes 5-1378 and 107-2049 t were

10 desiccated with the HRHT treatment and stored at 4°C. Embryos were subsequently removed from storage and nutrimprimed for one of 3, 4, or 5 days periods as previously described in ½ m24GMD liquid medium containing one of the following sucrose concentrations; 4, 6, 8, and 10% (all w/v). After the nutrimpriming treatments were completed, the embryos were sown ex vitro onto a

15 peat-based polymer-bound horticultural growing substrate in 504-cell (4 ml/cell) styrofoam miniplug trays (Grow-Tech Inc., San Bautista, CA). The sown trays were then placed into a conventional non-sterile horticulture germination chamber in an environment-controlled growth room. The environmental conditions were 22-24°C air temperature, 80-100% RH, 0.10 - 0.15% CO<sub>2</sub>, and a

20 16-hr photoperiod with 40-120 µmol m<sup>-2</sup>s<sup>-1</sup> photosynthetic photon flux. Immediately after the trays containing ex vitro sown nutrimprimed embryos were placed into the non-sterile germination chamber, they were misted with a ½ m24GMD nutrient solution amended with 3% w/v sucrose solution until the

surfaces of the non-sterile growing substrates were well-wetted. Subsequently, the trays were misted with this solution daily for a 3-week germination period. Aseptic techniques or conditions were not used for application of the nutrient solutions, nor for the germination environment during the 2-week culture period.

5 Furthermore, prophylactic applications of 0.1 g/l benlate, 0.05g/l ampicillin plus 0.05g/l penicillin G (or 0.1 ampicillin), and 5 ml/l Gnatrol® were applied at 5-day intervals to control fungi, bacteria, and fungus fly larvae.

The data in Tables 12.1 and 12.2 demonstrate that the range of sucrose concentrations that can be used for nutriming somatic embryos can extend up

10 to at least 10% and also, that the duration of nutriming can be increased to at least 5 days.



**Table 12.1:** Effects of increased sucrose concentrations and extended nutrimpriming duration on subsequent *ex vitro* germination and growth of somatic embryos from interior spruce genotype

5-1378.

5

% sucrose in nutrimpriming solutions	Duration of priming (days)	Germination %	Rooting %
3	3	83.3±8.8	73.3±8.8
4	3	98.3±1.7	86.7±7.3
	4	96.7±3.3	91.7±4.4
	5	96.5±1.8	91.5±6.0
6	3	91.7±4.4	86.7±3.3
	4	96.7±3.3	90.0±2.9
	5	98.3±1.7	88.3±4.4
8	3	91.7±4.4	66.7±12.0
	4	100	91.7±3.3
	5	95.0±5.0	86.7±8.3
10	3	84.7±2.9	52.5±7.2
	4	100	88.3±6.0
	5	98.3±1.7	91.7±4.4

**Table 12.2:** Effects of increased sucrose concentrations and extended nutrimpriming duration on subsequent *ex vitro* germination and growth of somatic embryos from interior spruce genotype 107-2049.

5

% sucrose in nutrimpriming solutions	Duration of priming (days)	Germination %	Rooting %
3	3	91.7±4.4	68.3±6.0
4	3	90.0±5.0	71.7±8.8
	4	84.6±6.2	62.3±13.0
	5	93.3±6.7	76.2±4.5
6	3	81.7±3.3	66.7±3.3
	4	85.8±2.2	69.6±6.1
	5	86.7±3.3	70.0±0.0
8	3	76.7±4.4	51.7±4.4
	4	93.3±4.4	88.3±4.4
	5	83.3±4.4	60.0±0.0
10	3	81.7±6.7	36.7±10.1
	4	90.0±7.6	76.7±8.3
	5	86.7±1.7	51.7±12.0

### EXAMPLE 13

The objective of this study was to assess the effects of nutrimpriming on the germination of an angiosperm plant species, *Brassica napus*.

- 10 Un-dessicated somatic embryos of canola cultivar Topas (*Brassica napus* cv. Topas) were nutrimprimed in 250-ml baffled Erlenmeyer culture flasks containing ½ m24GMD liquid medium supplemented with 3% w/v sucrose plus 80 units ml<sup>-1</sup> penicillin G, for a period of 7 days on a shaker table (80 rpm) in a controlled environment room kept at a temperature ranging between 20 –23°C
- 15 and with a photoperiod of 20–40 μmol m<sup>-2</sup>s<sup>-1</sup> photosynthetic photon flux. After

completion of the nutriming step, the nutrimed canola embryos were sown *ex vitro* onto 400-cavity styrofoam miniplug trays filled with peat bound by a polymer (Grow-Tech Inc., San Bautista, CA). After sowing, the trays were placed into a non-sterile high-humidity ( $\geq 95\%$  RH) germination chamber for one week.

- 5 In both studies, immediately after the trays containing *ex vitro* sown nutrimed embryos were placed into the non-sterile high-humidity germination chamber, they were misted with  $\frac{1}{2}$  m24GMD nutrient solution amended with 3% w/v sucrose once daily for five days. Thereafter, a  $1\text{g l}^{-1}$  of 11-41-8 fertilizer (Plant Products Co. Ltd., Brampton, ON) solution was applied three times a week for the
- 10 duration of the 3-week germination period. Aseptic techniques or conditions were not used for application of the nutrient solutions, or for the germination and growing environments during the 3-week culture period. At the completion of the 3-week period, 97.3% of the *ex vitro* sown nutrimed canola somatic embryos germinated in non-sterile growing mix in a non-sterile growing environment.

- 15 Mature somatic embryos of canola cultivar Topas (*Brassica napus* cv. Topas), were desiccated for a 3-week period using the HRHT treatment. After desiccation was completed, the somatic embryos were nutrimed in 250-ml baffled Erlenmeyer culture flasks containing  $\frac{1}{2}$  m24GMD liquid medium supplemented with 3% w/v sucrose plus 80 units  $\text{ml}^{-1}$  penicillin G, for a period of
- 20 7 days on a shaker table (80 rpm) in a controlled environment room kept at a temperature ranging between 20 –23°C and with a photoperiod of 20–40  $\mu\text{mol m}^{-2}\text{s}^{-1}$  photosynthetic photon flux. After completion of the nutriming step, the nutrimed canola embryos were sown *ex vitro* onto 400-cavity styrofoam miniplug trays filled with peat bound by a polymer (Grow-Tech Inc., San Bautista,
- 25 CA). After sowing, the trays were placed into a non-sterile high-humidity ( $\geq 95\%$  RH) germination chamber for one week. In both studies, immediately after the trays containing *ex vitro* sown nutrimed embryos were placed into the non-sterile high-humidity germination chamber, they were misted with  $\frac{1}{2}$  m24GMD nutrient solution amended with 3% w/v sucrose once daily for five days.
- 30 Thereafter, a  $1\text{g l}^{-1}$  of 11-41-8 fertilizer (Plant Products Co. Ltd., Brampton, ON) solution was applied three times a week for the duration of the 3-week

germination period. Aseptic techniques or conditions were not used for application of the nutrient solutions, or for the germination and growing environments during the 3-week culture period. At the completion of the 3-week period, 96.5% of the desiccated then nutriprimed, canola somatic embryos  
5 germinated when sown *ex vitro* into a non-sterile growing mix and maintained in a non-sterile growing environment.

Un-desiccated somatic embryos of canola cultivar Topas (*Brassica napus* cv. Topas) were nutriprimed in 250-ml baffled Erlenmeyer culture flasks containing  $\frac{1}{2}$  m24GMD liquid medium supplemented with 3% w/v sucrose plus 80  
10 units  $\text{ml}^{-1}$  penicillin G, for a period of 7 days on a shaker table (80 rpm) in a controlled environment room kept at a temperature ranging between 20 –23°C and with a photoperiod of 20-40  $\mu\text{mol m}^{-2}\text{s}^{-1}$  photosynthetic photon flux. After completion of the 1-week nutripriming step, the nutriprimed canola embryos were desiccated with a 1-week HRHT treatment and then separated into three groups.  
15 Group 1 was further desiccated in a 92.4% RH environment, Group 2 was desiccated in an 88.5% RH environment while Group 3 was desiccated in and 85% RH environment. Each group was desiccated for 3 days after which subsamples were removed for moisture content determinations. After the desiccation treatments were completed, the nutriprimed then desiccated canola  
20 somatic embryos were sown *ex vitro* onto 400-cavity styrofoam miniplug trays filled with peat bound by a polymer (Grow-Tech Inc., San Bautista, CA). After sowing, the trays were placed into a non-sterile high-humidity ( $\geq 95\%$  RH) germination chamber for one week. In both studies, immediately after the trays containing *ex vitro* sown nutriprimed embryos were placed into the non-sterile  
25 high-humidity germination chamber, they were misted with  $\frac{1}{2}$  m24GMD nutrient solution amended with 3% w/v sucrose once daily for five days. Thereafter, a  $1\text{ g l}^{-1}$  of 11-41-8 fertilizer (Plant Products Co. Ltd., Brampton, ON) solution was applied three times a week for the duration of the 3-week germination period. Aseptic techniques or conditions were not used for application of the nutrient  
30 solutions, or for the germination and growing environments during the 3-week culture period. The data recorded in Table 13 demonstrate that nutriprimed

canola somatic embryos can be desiccated prior to *ex vitro* sowing and germination.

Table 13: Effects of post-nutripriming desiccation on  
*ex vitro* germination of canola somatic embryos.

5

Replicate #	Desiccation treatment	Water content (%)	Germination %
1	92.4% RH	23.2±0.4	96.3
	88.5% RH	15.4±0.7	62.5
	85.0% RH	13.1±0.4	22.2
3	92.4% RH	20.5±0.5	85.7
	88.5% RH	15.5±0.4	35.7
	85.0% RH	14.5±0.5	26.2
4 <sup>1)</sup>	92.4% RH	17.6±0.6	76.7
	88.5% RH	13.4±0.1	40.0
	85.0% RH	12.7±1.1	43.3

1) Germination percentages were recorded 2 weeks after sowing.

## REFERENCES

1. Bradford, K.J. (1986) Manipulation of seed water relation via osmotic priming to improve germination under stress conditions. *HortScience* 21: 1105-1112.
2. Carlson, W.C. and J.E. Hartle. (1995) Manufactured Seeds of Woody Plants. *IN* Somatic Embryogenesis of Woody Plants. Vol. I. S.M. Jain, P.K. Gupta, and R.J. Newton (Eds.) Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 253-263.
3. Feirer, RP, Conkey, J.H., Verhagen, S.A. 1989. Triglycerides in embryogenic conifer calli: a comparison with zygotic embryos. *Plant Cell Reports* 8: 207-209.
4. Gupta, P. and J.A. Grob. (1995) Somatic Embryogenesis in Conifers. *IN* Somatic Embryogenesis of Woody Plants. Vol. I. S.M. Jain, P.K. Gupta, and R.J. Newton (Eds.) Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 81-98.
5. Heydecker, W. and Coolbear P. (1977) Seed treatment for improved performance-survey and attempt prognosis. *Seed Science and Technology* 5: 353-425.
6. Roberts, D.R., F.B. Webster, B.S. Flinn, W.R. Lazaroff & D.R. Cyr. 1993. Somatic embryogenesis of spruce *IN*: K. Redenbaugh (Ed.) *Synseeds: Application of synthetic seeds to crop improvement*. CRC Press, Boca Raton FL. Pp. 427 - 452.
7. McDonald, M.B. 2000 Seed priming *IN*: *Seed Technology and Its Biological Basis*. M. Black & J.D. Bewley (Eds.) Sheffield Academic Press (in press).
8. Roberts. D.R. F.B. Webster, D.R. Cyr, T.K. Edmonds, S.M.A. Grimes & B.C.S. Sutton. (1995) *IN*: J. Aitken-Christie, T. Kozai, and M.A.L. Smith (Eds.) *Automation and Environmental Control in Plant tissue Culture* Kluwer Academic Pub. Dordrecht, The Netherlands. pp. 245 - 256.
9. Sakamoto, Y., N. Onishi, and T. Hirose. (1995) Delivery Systems for Tissue Culture by Encapsulation. *IN* *Automation and Environmental Control in Plant Tissue Culture*. J. Aitken-Christie, T. Kozai, and M.L.A. Smith, Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 215-243.
10. Simon, E.W. (1984) Early events in germination. *In* *Seed Physiology* (D.R. Murray ed.) Academic Press. Toronto. pp 77-115.

## CLAIMS:

1. A process of nutripriming plant somatic embryos prior to sowing, characterized in that imbibed plant somatic embryos are contacted with a solution containing a dissolved nutrient.
2. A process according to claim 1, characterized in that said nutrient is a carbohydrate.
3. A process according to claim 1, characterized in that said nutrient is a sugar.
4. A process according to claim 1, characterized in that said nutrient is sucrose.
5. A process according to claim 4, characterized in that said sucrose is present in said solution in an amount of 6% w/v or less.
6. A process of nutripriming plant somatic embryos prior to germination, characterized in that imbibed plant somatic embryos are contacted with a solution comprising a mixture of two or more dissolved nutrients.
7. A process according to claim 6, characterized in that one of said nutrients is a sugar.
8. A process according to claim 6, characterized in that one of said nutrients is sucrose.
9. A process according to claim 8, characterized in that said sucrose is present in said solution in an amount of 6% w/v or less.
10. A process according to claim 6, claim 7, claim 8 and claim 9, characterized in that one of said nutrients is an amino acid.
11. A process according to any one of claims 6 to 10, characterized in that one of said nutrients is a nitrate ion.
12. A process according to any one of claims 6 to 11, characterized in that one of said nutrients is an ammonium ion.
13. A process according to any one of claims 6 to 12, characterized in that one of said nutrients is a phosphate ion.
14. A process according to any one of claims 6 to 13, characterized in that one of said nutrients is a potassium ion.
15. A process according to any one of claims 6 to 14, characterized in that one of said nutrients is a micronutrient.
16. A process according to any one of claims 6 to 15, characterized in that one of said nutrients is a vitamin.
17. A process according to any one of claims 6 to 16, characterized in that one of said nutrients is a plant growth regulator.

18. A process according to any preceding claim, characterized in that said plant somatic embryo is from a tree species.
19. A process according to any preceding claim, characterized in that said plant somatic embryo is from a gymnosperm.
20. A process according to any preceding claim, characterized in that said plant somatic embryo is a spruce embryo.
21. A process according to any preceding claim, characterized in that said plant somatic embryo is an interior spruce embryo.
22. A process according to any one of claims 1 to 20, characterized in that said plant somatic embryo is a pine embryo.
23. A process according to claim 21, characterized in that said plant somatic embryo is a loblolly pine embryo.
24. A process according to claim 22, characterized in that said plant somatic embryo is a radiata pine embryo.
25. A process according to claim 22, characterized in that said plant somatic embryo is a *Pinus patula* embryo.
26. A nutrimed somatic embryo characterized in that the embryo is produced by a process according to any preceding claim.
27. A method of producing seedlings or full-grown plants from somatic embryos, which comprises germinating imbibed somatic embryos in a growth medium to form germinants, and maintaining growing conditions to allow the germinants to grow into seedlings or full-grown plants, characterized in that said imbibed somatic embryos are nutrimed by contacting said imbibed somatic embryos with a solution containing a dissolved nutrient.
28. A method according to claim 27, characterized in that a nutrient is incorporated into said growth medium.
29. A method according to claim 28, characterized in that the nutrient incorporated into the growth medium is the same as the nutrient used in the nutriming step.
30. A method according to claim 27, claim 28, or claim 29, characterized in that said germinating of the nutrimed embryos and said growing of said germinants are carried out in non-sterile conditions.
31. A method according to any one of claims 27 to 30, characterized in that the nutrient used in the nutriming step is a carbohydrate.
32. A method according to any one of claims 27 to 31, characterized in that the nutrient used in the nutriming step is a sugar.



33. A method according to any one of claims 27 to 32, characterized in that the nutrient used in the nutriming step is sucrose.
34. A method according to claim 33, characterized in that sucrose is present in said growth medium in an amount of 6% w/v or less.
35. A method according to any one of claims 27 to 34, characterized in that the plant somatic embryo is from a tree species.
36. A method according to any one of claims 27 to 35, characterized in that the plant somatic embryo is from a gymnosperm.
37. A method according to any one of claims 27 to 36, characterized in that the plant somatic embryo is a spruce embryo.
38. A method according to any one of claims 27 to 37, characterized in that the plant somatic embryo is an interior spruce embryo.
39. A method according to any one of claims 27 to 36, characterized in that the plant somatic embryo is a pine embryo.
40. A method according to claim 39, characterized in that the plant somatic embryo is a loblolly pine embryo.
41. A method according to claim 39, characterized in that the plant somatic embryo is a radiata pine embryo.
42. A method according to claim 39, characterized in that the plant somatic embryo is a *Pinus patula* embryo.
43. Seedlings or full-grown plants, characterized in that said seedling or plants are produced by a method according to any one of claims 27 to 42.
44. A method of producing seedlings or full-grown plants from somatic embryos, which comprises germinating imbibed somatic embryos in a growth medium to form germinants, and maintaining growing conditions to allow the germinants to grow into seedlings or full-grown plants, characterized in that the imbibed somatic embryos are nutrimed by contacting said imbibed embryos with a solution containing a mixture of dissolved nutrients.
45. A method according to claim 44, characterized in that a nutrient is incorporated into said growth medium.
46. A method according to claim 45, characterized in that the nutrient incorporated into the growth medium is the same as the nutrient used in the priming step.
47. A method according to claim 44, claim 45 or claim 46 characterized in that said germinating of the nutrimed embryos and said growing of said germinants are carried out in non-sterile conditions.

48. A method according to claim 45 or 46, characterized in that the nutrient incorporated into said growth medium is a carbohydrate.
49. A method according to claim 45 or 46, characterized in that the nutrient incorporated into said growth medium is a sugar.
50. A method according to claim 45 or 46, characterized in that the nutrient incorporated into said growth medium is sucrose.
51. A method according to claim 50, characterized in that sucrose is present in said growth medium in an amount of 6% w/v or less.
52. A method according to any one of claims 44 to 51, characterized in that the plant somatic embryo is from a tree species.
53. A method according to any one of claims 44 to 52, characterized in that the plant somatic embryo is from a gymnosperm.
54. A method according to any one of claims 44 to 53, characterized in that the plant somatic embryo is a spruce embryo.
55. A method according to any one of claims 44 to 54, characterized in that the plant somatic embryo is an interior spruce embryo.
56. A method according to any one of claims 44 to 53, characterized in that the plant somatic embryo is a pine embryo.
57. A method according to claim 56, characterized in that the plant somatic embryo is loblolly pine embryo.
58. A method according to claim 56, characterized in that the plant somatic embryo is a radiata pine embryo.
59. A method according to claim 56, characterized in that the plant somatic embryo is a *Pinus patula* embryo.
60. Seedlings or full-grown plants, characterized in that said seedlings or plants are produced by a method according to any one of claims 44 to 59.
61. A method of germinating mature plant somatic embryos, characterized by priming imbibed embryos in the presence of a nutrient solution.
62. A method according to claim 61, characterized in that said nutrient is selected from the class comprising carbohydrates.
63. A method according to claim 61, characterized in that said nutrient is selected from the class comprising sugars.
64. A method according to claim 61, characterized in that said nutrient is sucrose.
65. A method according to claim 64, characterized in that sucrose is present in said nutrient solution in a concentration of no greater than about 6% w/v.

66. A method according to claim 64, characterized in that said nutrient is selected from the class comprising amino acids.
67. A method according to any one of claims 61 to 66, characterized in that at least one additional nutrient is a nitrate ion.
68. A method according to any one of claims 61 to 67, characterized in that at least one additional nutrient is an ammonium ion.
69. A method according to any one of claims 61 to 68, characterized in that at least one additional nutrient is a phosphate ion.
70. A method according to any one of claims 61 to 69, characterized in that at least one additional nutrient is a potassium ion.
71. A method according to any one of claims 61 to 70, characterized in that at least one additional nutrient is selected from the class comprising micronutrients.
72. A method according to any one of claims 61 to 71, characterized in that at least one additional nutrient is selected from the class comprising vitamins.
73. A method according to any one of claims 61 to 72, characterized in that the nutrient medium further comprises a plant growth regulator.
74. A method according to any one of claims 61 to 73, characterized in that the plant somatic embryo is from a tree species.
75. A method according to any one of claims 61 to 74, characterized in that the plant somatic embryo is from a gymnosperm.
76. A method according to any one of claims 61 to 75, characterized in that the plant somatic embryo is a spruce embryo.
77. A method according to any one of claims 61 to 76, characterized in that the plant somatic embryo is an interior spruce embryo.
78. A method according to any one of claims 61 to 75, characterized in that the plant somatic embryo is a pine embryo.
79. A method according to claim 78, characterized in that the plant somatic embryo is loblolly pine embryo.
80. A method according to claim 78, characterized in that the plant somatic embryo is a radiata pine embryo.
81. A method according to claim 78, characterized in that the plant somatic embryo is a *Pinus patula* embryo.
82. Germinated mature plant somatic embryos characterized in that said embryos have been produced by a process according to any one of claims 61 to 81.

83. A method of producing somatic seedlings, characterized by (i) priming imbibed mature plant somatic embryos in a nutrient medium, and (ii) sowing the embryos in a sowing medium.
84. A method according to claim 83, further comprising applying nutrients to the sown embryos.
85. A method according to claim 84, characterized in that the means of applying nutrients to the sown embryos is selected from the group comprising spraying, misting, drenching, and irrigating.
86. A method according to claim 83, claim 84 or claim 85, characterized in that the plant somatic embryo is from a tree species.
87. A method according to claim 83, claim 84 or claim 85 characterized in that the plant somatic embryo is from a gymnosperm.

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/CA 00/00411

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 A01H4/00 A01C1/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 A01H A01C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, PAJ, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 11660 A (UNIV SASKATCHEWAN) 24 June 1993 (1993-06-24)	1-4, 6-8, 18-22, 27, 31-33, 35-39, 43, 44, 48-50, 52-56, 60-64, 74-78, 82-87
Y	page 40, last paragraph -page 43, last paragraph	5, 9-17, 34, 51, 65-73
Y	US 5 563 061 A (GUPTA PRAMOD K) 8 October 1996 (1996-10-08) the whole document	10-17, 66-73

☒ Further documents are listed in the continuation of box C.

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# INTERNATIONAL SEARCH REPORT

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 57536 A (UNIV SASKATCHEWAN) 23 December 1998 (1998-12-23) claims ---	5,9,34, 51,65
A	WO 96 37095 A (CARTER HOLT HARVEY LTD ;AITKEN CHRISTIE JENNIFER (NZ); GOUGH KEIKO) 28 November 1996 (1996-11-28) the whole document ---	1-87
A	US 5 464 769 A (ATTREE STEPHEN M ET AL) 7 November 1995 (1995-11-07) cited in the application the whole document ---	1-87
A	US 5 677 185 A (HANDLEY III LEVIS W) 14 October 1997 (1997-10-14) the whole document ---	1-87
A	US 4 780 987 A (NELSEN CHARLES ET AL) 1 November 1988 (1988-11-01) the whole document ---	1-87
A	US 5 482 857 A (GUPTA PRAMOD K ET AL) 9 January 1996 (1996-01-09) cited in the application the whole document ---	1-87
A	US 5 491 090 A (HANDLEY III LEVIS W ET AL) 13 February 1996 (1996-02-13) the whole document -----	1-87

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 00/00411

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9311660	A	24-06-1993	AU 679435 B AU 3154193 A CA 2125410 A CA 2238373 A EP 0618766 A FI 942909 A NO 942324 A NZ 246137 A US 5464769 A US 5985667 A	03-07-1997 19-07-1993 24-06-1993 24-06-1993 12-10-1994 17-08-1994 17-08-1994 26-04-1996 07-11-1995 16-11-1999
US 5563061	A	08-10-1996	US 5294549 A US 5236841 A US 5036007 A US 4957866 A US 5034326 A AU 700663 B AU 5481096 A CA 2221610 A NZ 306279 A WO 9637097 A AU 1293495 A WO 9514373 A ZA 9409308 A AU 680206 B AU 5081393 A WO 9505070 A US 5482857 A AU 654939 B AU 6641290 A AU 672531 B AU 7585294 A CA 2069964 A,C NZ 235795 A NZ 245524 A WO 9105854 A AU 619392 B AU 5161390 A CA 2028855 A,C EP 0423260 A NZ 232834 A WO 9010382 A US 5041382 A	15-03-1994 17-08-1993 30-07-1991 18-09-1990 23-07-1991 14-01-1999 11-12-1996 28-11-1996 28-10-1999 28-11-1996 13-06-1995 01-06-1995 08-08-1995 24-07-1997 14-03-1995 23-02-1995 09-01-1996 01-12-1994 16-05-1991 03-10-1996 16-03-1995 24-04-1991 28-04-1993 26-05-1993 02-05-1991 23-01-1992 09-10-1990 10-09-1990 24-04-1991 27-01-1993 20-09-1990 20-08-1991
WO 9857536	A	23-12-1998	AU 8006298 A EP 0987937 A	04-01-1999 29-03-2000
WO 9637095	A	28-11-1996	NZ 272210 A AU 699907 B AU 5241896 A CA 2221720 A ZA 9604104 A	24-10-1997 17-12-1998 05-12-1996 28-11-1996 03-12-1996
US 5464769	A	07-11-1995	AU 679435 B AU 3154193 A CA 2125410 A CA 2238373 A WO 9311660 A	03-07-1997 19-07-1993 24-06-1993 24-06-1993 24-06-1993

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. .ional Application No

PCT/CA 00/00411

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5464769 A		EP 0618766 A	12-10-1994
		FI 942909 A	17-08-1994
		NO 942324 A	17-08-1994
		NZ 246137 A	26-04-1996
		US 5985667 A	16-11-1999
US 5677185 A	14-10-1997	AU 689407 B	26-03-1998
		AU 2014697 A	20-11-1997
		BR 9703137 A	18-08-1998
		CA 2205188 A	14-11-1997
		NZ 314791 A	29-06-1999
		US 5856191 A	05-01-1999
US 4780987 A	01-11-1988	US 4583320 A	22-04-1986
		AT 61902 T	15-04-1991
		AU 608743 B	18-04-1991
		AU 6339486 A	24-03-1987
		CA 1282609 A	09-04-1991
		CN 86106145 A	03-06-1987
		DE 3678427 D	02-05-1991
		EP 0236444 A	16-09-1987
		JP 63500911 T	07-04-1988
		NZ 217493 A	21-12-1990
		WO 8701258 A	12-03-1987
		AT 68316 T	15-11-1991
		AU 1702592 A	10-09-1992
		AU 2699188 A	20-04-1989
		AU 3464284 A	02-05-1985
		CA 1251335 A	21-03-1989
		DE 3485173 A	21-11-1991
		EP 0141373 A	15-05-1985
		JP 1782526 C	13-08-1993
		JP 4069970 B	09-11-1992
		JP 60214811 A	28-10-1985
		US 4779376 A	25-10-1988
US 5482857 A	09-01-1996	US 5294549 A	15-03-1994
		US 5236841 A	17-08-1993
		US 5036007 A	30-07-1991
		US 4957866 A	18-09-1990
		US 5034326 A	23-07-1991
		CA 2158984 A	31-08-1995
		NZ 281862 A	24-10-1997
		WO 9522887 A	31-08-1995
		AU 680206 B	24-07-1997
		AU 5081393 A	14-03-1995
		WO 9505070 A	23-02-1995
		US 5563061 A	08-10-1996
		AU 654939 B	01-12-1994
		AU 6641290 A	16-05-1991
		AU 672531 B	03-10-1996
		AU 7585294 A	16-03-1995
		CA 2069964 A,C	24-04-1991
		NZ 235795 A	28-04-1993
		NZ 245524 A	26-05-1993
		WO 9105854 A	02-05-1991
		AU 619392 B	23-01-1992
		AU 5161390 A	09-10-1990



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 00/00411

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5482857 A		CA 2028855 A, C EP 0423260 A NZ 232834 A WO 9010382 A US 5041382 A	10-09-1990 24-04-1991 27-01-1993 20-09-1990 20-08-1991
US 5491090 A	13-02-1996	AU 695091 B AU 1484295 A BR 9501707 A NZ 270706 A ZA 9502325 A	06-08-1998 29-08-1996 12-08-1997 28-10-1996 03-01-1996